NASA Technical Memorandum 86043

Planetary Biology and Microbial Ecology

Biochemistry of Carbon and Early Life

Edited by
Lynn Margulis
Kenneth H. Nealson
Isobel Taylor

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Edited by
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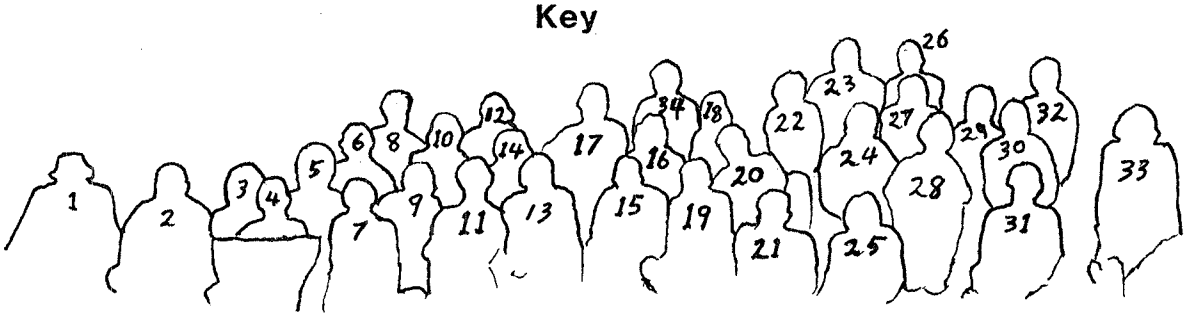

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Note to reader:

Each lecturer supplied key references to document his presentation. These are listed on pages xxiv to xxxviii. References called out in each of the chapters can be found at the end of each chapter. Technical information pertaining to cyanobacteria, phototrophic bacteria and methanogens can be found in appendices to Chapters I, II, and III respectively. There are also appendices at the end of the entire report which deal with isotope analyses and techniques. Each appendix is followed by specific references to technique.

Page ii designed by Linda Angeloff Sapienza
Photo, page 135 -- Apollo 8, view of the Earth from 280,000 miles away

PLANETARY BIOLOGY AND MICROBIAL ECOLOGY:
BIOCHEMISTRY OF CARBON AND EARLY LIFE

INTRODUCTION

CHAPTER I. CYANOBACTERIA AND THE CARBON CYCLE

CHAPTER II. ANOXYGENIC PHOTOTROPHIC BACTERIA

CHAPTER III. ECOLOGY OF METHANOGENESIS

LIST OF FIGURES

LIST OF TABLES

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We know you will understand that the delay in appearance of this manuscript was due to the innumerable tasks related to its completion. That it has appeared at all is because of the willingness of the NASA printing office, Bill Dalton, Kay Voglewede, Faye Ellen, and Wayne Williams to print and bind it.

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Planetary Biology
Microbial Ecology
Program

June 21--July 30, 1982

Students arrived June 20; faculty arrived June 19.

Week 1

June 21, Monday

8:30 AM--12PM Introduction: Kenneth Nealson
Short presentations by L. Baresi, D. DesMarais, W. Krumbein, H.Trueper; students introduced themselves
1:30 PM Introductory lectures:
G. Tomlinson, procaryote structure; K. Nealson, procaryote physiology. Permanent faculty and students organized lab equipment.
8:00 PM Stable isotope geochemistry: D. DesMarais.

June 22, Tuesday

8:30 AM Phototrophic bacteria: H. Trueper, West Germany
11:00 AM--4:30 PM Lunch at NASA Ames and tour
1:00 PM Overview of NASA Ames: S. Mead
1:30 PM Overview of Ames Life Sciences: H. Klein
2:00 PM Aviation human factors research: C. Billings
2:30 PM Weightlessness and bedrest: J. Billingham
3:00 PM Vestibular physiology and space: P. Cowings
3:30 PM Motion sickness: J. Billingham
4:00 PM Controlled ecological life support systems: R. McElroy
7:00 PM Program reception at the home of J. Billingham

June 23, Wednesday

8:30 AM Cyanobacteria: W. Krumbein
11:00 AM--5:00 PM Field trip to Alum Rock Park for sample collection.

June 24, Thursday

8:30 AM Methanogenesis: L. Baresi
11:00 AM--3:00 PM Field trip to Palo Alto Baylands and salt ponds for sample collection.
8:00 PM Organization of research groups

June 25, Friday

Lab group meetings; projects begun

June 26, Saturday

Lab projects continued. Visiting faculty arrived: Drs. Mary Lidstrom, J. Gregg Ferry, J. Kasting, and William Reeburgh.

June 27, Sunday

8:00 PM Reception for visiting faculty

June 28--July 4
Week 2

Lecture 1: 8:30 AM	**Lecture 2: 10:30 AM**
June 28, Monday	
Early history of the atmosphere: J. Kasting	Methanogenic bacteria I: J. G. Ferry
June 29, Tuesday	
Environmental rates of methane production: W. Reeburgh	Methanogenic bacteria II: J.G. Ferry
June 30, Wednesday	
Environmental rates of methane consumption: W. Reeburgh	Methylotrophs I: M. Lidstrom
July 1, Thursday	
Methylotrophs II: M. Lidstrom	Anaerobic methane consumption: W. Reeburgh

July 2, Friday

Geochemistry of Big Soda Lake:
R. Oremland

Origin of atmospheric O_2:
J. Kasting

July 3, Saturday

Visitors arrived: Drs. W. Broecker, J. Hayes, A. Knoll, and B. Pierson.

8:00 PM Comparative Planetary Atmospheres: Dr. J. Kasting

July 4, Sunday

Holiday--Lab barbecue

July 5--12
Week 3

July 5, Monday

Carbon Cycling:
W. Broecker

Phototrophs I:
B. Pierson

8:00 PM Round table review of previous week's material

July 6, Tuesday

Evolution of phototrophs:
H. Trueper

Fossil microbial communities:
A. Knoll

July 7, Wednesday

Organic geochemistry I:
J. Hayes

Chemoautotrophy:
A. Matin

July 8, Thursday

Carbon in the oceans:
W. Broecker

Phototrophs II:
B. Pierson

July 9, Friday

PrePhanaerozoic fossil record: A. Knoll

Organic Geochemistry II: J. Hayes

July 10, Saturday

Visitors arrived: Drs. S. Awramik, R. Garrels, C. Sapienza

8:00 PM Origins of Life: Dr. Sherwood Chang.

July 11, Sunday

Field trip to Point Reyes for interested program participants.

July 12--18
Week 4

July 12, Monday

Modern and fossil microbial communities: Lynn Margulis

Symbiosis and Cell Evolution: L. Margulis

7:00 PM Films--*Polyangium*, *Labyrinthula*, spirochetes, sex in protists, chloroplasts, *Microcoleus*, Bitter Springs fossils: L. Margulis

8:00 PM Round table review of previous week's material.

July 13, Tuesday

Evolution of genomes: C. Sapienza

Archaebacterial genomes: C. Sapienza

July 14, Wednesday

Sedimentary systems of the Earth: R. Garrels

A chemist's view of biogeochemical zonation: C. Martens

8:00 PM Introduction to the Gaia hypothesis: L. Margulis

July 15, Thursday

In situ methane measurements--Cape Lookout Bight: Chris Martens

History of global CO_2, oxygen, silica and carbonate rocks: R. Garrels

8:00 PM Round table review of atmospheric, ocean and sedimentary history: W. Broecker, R. Garrels, J. Kasting, C. Martens, L. Margulis

July 16, Friday

Gunflint microbiota: S. Awramik

Stromatolites and potential stromatolites: W. Krumbein

4:00--8:00 PM NASA Extraterrestrial picnic, John Billingham, host.

July 17, Saturday

8:30 AM PrePhanerozoic stromatolites and stratigraphy: S.M. Awramik
9:30 AM Carbon isotope fractionation in deep sea vents and upland lakes: G. Rau.
7:00 PM Intramolecular isotope fractionation in glycolysis by *Escherichia coli*: N. Blair
8:00 PM Manganese cycling--bacterial contributions: K. Nealson
9:00 PM Stratified microbial communities: W. Krumbein

July 18, Sunday

PrePhanerozoic microfossils from China: S. Awramik

July 19--24
Week 5

July 19, Monday

8:00 PM Research report meeting

July 20--23, Tuesday through Friday

Research teams continue laboratory work

July 24, Saturday

8:00 PM The Gaia hypothesis revisited: L. Margulis

July 26--July 31
Week 6

July 28, Wednesday

Bacterial luminescence: K. Nealson

July 29, Thursday

5:00 PM Barbecue, Graham Pool

July 30, Friday

1:00 PM Student presentations
5:00 PM Mexican dinner at the home of G. Tomlinson

July 31, Saturday

Departure of participants

Stanley M. Awramik: ANCIENT MICROBIAL COMMUNITIES AND STROMATOLITES

Awramik, S.M., 1981. The pre-Phanerozoic biosphere - three billion years of crises and opportunities, pp. 83-102. In *Biotic Crises in Ecological and Evolutionary Time*, (M. H. Nitecki, ed.), Academic Press, New York.

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Wallace Broecker: CARBON CYCLING

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David DesMarais: STABLE ISOTOPES

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Robert Garrels: ATMOSPHERIC EFFECTS ON THE EARTH

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THE RELATION OF THE PLANETARY BIOLOGY AND MICROBIAL ECOLOGY COURSE TO NASA AMES RESEARCH CENTER AND THE UNIVERSITY OF SANTA CLARA

In 1980 the PBME course held nearly its entire lecture program at NASA Ames and nearly its entire laboratory program at the Biology Department of the University of Santa Clara. In fact, in 1980, very few NASA scientists attended PBME lectures and much time and energy was wasted on the freeway. Furthermore, access to NASA's scientific expertise and special technology was very limited. In 1982, in response to the suggestions received, primarily by a questionnaire sent to all participants of the 1980 program, the relationship between the PBME and NASA was vastly improved. One of the concerns expressed by respondents to the questionnaire was the problem of continuity. Although unfortunately no formal mechanism to insure continued collaboration between PBME students and NASA was worked out, several participants in the 1980 course are continuing work begun in the program (e.g., David Burdige, James Cowen, Betsey Grosovsky, and John Stolz all presented papers at the Fifth International Meeting on Biomineralization and Biological Metal Deposition, at Renesse, The Netherlands, organized by Prof. Peter Westbroek, also a 1980 PBME participant. Prof. Antonio Lazcano-Araujo, together with Celia Ramirez gave a new summer course, in 1981 on the origin of life and microbial ecology. Lazcano also presented a short course on this subject at the University of Alicante, Spain, 1981.)

The major scientific project of the summer of 1982, measurements of isotope fractionation by autotrophic microorganisms in the field and in the laboratory, was an entirely joint project of NASA Ames and the course. David DesMarais, as the leader of the carbon isotope fractionation research effort, provided far more than access to the GCMS equipment. The research goals of the PBME program and those of DesMarais were the same. Because DesMarais had planned and coordinated all aspects of the mass spectrometry preparation and measurement, more information may have been collected and exchanged about microbial fractionation of carbon isotopes during the summer PBME program than previously in the entire experience of microbiogeochemistry. Furthermore the students had opportunities to learn advanced techniques and use state of the art equipment; such opportunities are generally unavailable in even the best equipped graduate schools.

In addition to the immeasurable contribution made by DesMarais to this program, and in spite of the fact that lectures were held at Santa Clara, NASA scientists participated regularly. Visiting and staff scientists at NASA (e.g., Gregory Rau, Neil Blair, James Lawless, James Kasting, Sherwood Chang) gave and attended scheduled lectures and joined in many other activities. Opportunities to visit various NASA laboratories and facilities were provided. For example, Dr. H. P. Klein provided an overview of NASA Ames Life Sciences, Dr. Billingham reviewed research in human factors in aviation and motion sickness, and Dr. McElroy presented a review of the CELSS (controled ecological life support systems) program. In summary, the partnership between PBME and NASA Ames was greatly improved in 1982. In planning for the 1984 PBME scientific research program we will continue to strengthen our relationship with ongoing NASA research.

CHAPTER I
CYANOBACTERIA AND THE CARBON CYCLE

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Margriet Nip
Kent Sprague
Maud Walsh
Kevin Zahnle

INTRODUCTION TO CYANOBACTERIA

Importance of Cyanobacteria

(Kevin Zahnle)

Cyanobacteria are the most widely distributed photosynthetic organisms, thriving in a wide range of temperature, salinity, and illumination. They are among the oldest of earth's inhabitants. Cyanobacteria probably first appeared in the fossil record as early as 3.5 billion years ago in the Warrawoona Supergroup, Western Australia. Much work on their history has depended on molecular biological studies of modern forms (Woese and Fox, 1977), but fossil bacteria and their residual organic matter can be studied directly (Hayes, *et al.*, in press). They (or their direct ancestors) are credited with the formation of oxygenic photosynthesis, a truly epochal event that revolutionized the biosphere. The use of abundant water as an electron donor in photosynthesis permitted biological access to environments that were previously forbidden for want of relatively scarce molecules such as H_2, H_2S, S^o and $S_2O_3^{-2}$ which probably were abundant only locally, rather than globally. The generation of oxygen as a byproduct spurred the development and radiation of aerobic respiration.

The generation of oxygen eventually led to other global consequences. Oxygen reacted with the crust--indeed, the reaction may have been the *genesis* of the continental crust. It is certainly responsible for forming large reservoirs of iron oxides and sulfates from previously reduced material (Broecker, 1970). Biogenic oxygen may even have altered the initial oxidation state of the entire upper mantle (Arculis and Delano, 1980). Eventually, oxygen generation overwhelmed the abiotic sources of reducing power and produced an oxygen-rich atmosphere which also heavily affected the evolution of life (Walker, 1980).

In keeping with the dominant theme of this project, *i.e.* the discrimination by living systems between the two stable carbon isotopes, we have focused much of our experimental work on the carbon isotope fractionation by cyanobacteria, both for its

intrinsic interest and as a research tool.

In general, biological matter is isotopically light, preferring to incorporate ^{12}C to ^{13}C. Most of this fractionation occurs in the uptake of CO_2 when used as a carbon source (Smith, 1972). For cyanobacteria, as for the C3 plants, this occurs in the carboxylation of ribulose bisphosphate (RuBP). Typically, C3 plants are isotopically light by some -25 °/₀₀, although there is a great deal of variation. Cyanobacteria are apparently somewhat heavier, with reported fractionations by laboratory-grown pure cultures ranging from -11 °/₀₀ to -24 °/₀₀ (Pardue, *et al.*, 1975). The extent to which they fractionate depends strongly on environmental factors. Conditions unfavorable to growth tend to cause more fractionation, although higher CO_2 concentrations also augment *delta* ^{13}C fractionation (Pardue, *et al.*, 1975). Interpretation of the data is further complicated by the closed system effect, in which a dense community becomes isotopically heavier as it makes use of carbon faster than it can be exchanged with the external world, thus causing the community to be living in a locally isotopically heavy carbon pool.

Partly because the early prePhanerozoic fossil record is so limited, the carbon isotope record as preserved and/or metamorphosed in fossil microbial mats has played a large role in the reconstruction of our hazy past. Typical prePhanerozoic reduced carbon is isotopically light, -30 °/₀₀ (Hayes *et al*, in press). Unfortunately, the isotope record has its pitfalls; not least among these is our inadequate knowledge of how modern microbial mats (thought by many to be directly analogous to the stromatolites that figure so prominently in the prePhanerozoic fossil record *e.g.*, Krumbein, 1979) fractionate as a function of depth, population density, nutrient availability, and state of degradation. Modern mats are less fractionated, about -16 °/₀₀, than the fossil sedimentary carbon to which they are usually compared (Pardue *et al.*, 1975). We studied isotope fractionation, correlated with microorganisms living in three modern microbial communities: two populations from lukewarm sulfur springs in Alum Rock Park, and one from a salt marsh in the Palo Alto Baylands adjacent to the San Francisco Bay.

We also used stable isotopes to study the use of glucose by an axenic culture of *Phormidium luridium*. This experiment demonstrates some of the power of stable isotopes as an analytical technique.

Although cyanobacteria are widely distributed and thrive in a wide range of environments, they do not seem to do very well in Petri dishes, bottles, and test tubes. Nevertheless, a major part

of our group effort was dedicated to the characterization, growth, isolation, and purification of diverse strains of cyanobacteria gathered at the three aforementioned sites.

The role of cyanobacteria in the global carbon cycle was examined in several different experiments. We studied the preservation potential and morphologic degradation stages of cyanobacteria and compared these to fossilized organisms, preserved mainly in cherts that resemble modern cyanobacterial communities.

The following projects were undertaken:

Comparison of ancient and recent microbial mats and the microorganisms within them.

Isolation and purification of 6-8 strains of cyanobacteria from Alum Rock Park.

Isolation and purification of 4-6 strains of cyanobacteria from the salt marshes at Embarcadero Road.

Detailed description of the microbiota of Alum Rock Park and the Embarcadero Road salt marshes, including:

Descriptions of cyanobacteria, anoxygenic photosynthetic bacteria, chemolithotrophic bacteria, and eukaryotes in both environments.

Measurement of chlorophyll/protein ratios per surface unit area.

Delta ^{13}C fractionation patterns from different communities of both environments.

Delta ^{13}C fractionation in cyanobacteria selected by motility experiments directly in the environment.

Delta ^{13}C fractionation by pure cultures of cyanobacteria *Phormidium*, *Oscillatoria*.

Nitrogen stable isotope fractionation patterns and nitrogen fixation by the Alum Rock and salt marsh communities.

Delta ^{13}C fractionation by pure cultures re-introduced into their natural environments (i.e., "natural chemostats"), kept physically, but not chemically, separated by dialysis tubing.

Description of partial degradation sequences of microorganisms for comparison with fossil microorganisms.

We attempted to study ^{13}C fractionation by an axenic culture of *Phormidium luridium* under the following nutritional modes:

Aerobic oxygenic photolithoautotrophic
Anaerobic oxygenic photolithoautotrophic
Anoxygenic photoorganoautotrophic
Anoxygenic photoorganoheterotrophic
Chemoorganoheterotrophic

COMMUNITY STRUCTURE AND FIELD SITES

(Margriet Nip and Maude Walsh)

Alum Rock Park

The warm sulfur springs of Alum Rock State Park are sites of varied microbial communities. Two springs were chosen for "natural chemostat" experiments where carbon isotopic fractionation of both the communities and specific members of the community could be isolated and studied.

Alum Rock Park Site 1 (Figs. I-1 and I-2) is a steep talus slope on the banks of Penitencia Creek, extending from the road south of the stream to the stream bed. The main components of the microbial community at this site were: purple (*Chromatium*) and green (*Chlorobium*) sulfur bacteria, *Thiothrix*, a colorless filamentous sulfur bacterium which deposits sulfur globules internally, cyanobacteria and green algae. The purple and green sulfur bacteria occurred in small patches (of a few cm^2 of surface area) near the top of the spring where the water first emerged from the rocks. *Thiothrix* predominated near the top of the flow, and followed the main water flow to near the edge of the slope. Extending outward from the concentration of *Thiothrix* were the dark green patches of cyanobacteria.

The cyanobacteria community was varied. In some areas there were small pillows (up to 1 cm diameter). The pillows were made up of a thin LPP Group A filament, an *Oscillatoria*, and a few *Calothrix*, with purple and green sulfur bacteria at the center (Fig. I-3-4). The cyanobacterial community also comprised white and dark green mats (less than 2 mm thick). The dark green

Figure I-1 Alum Rock Park

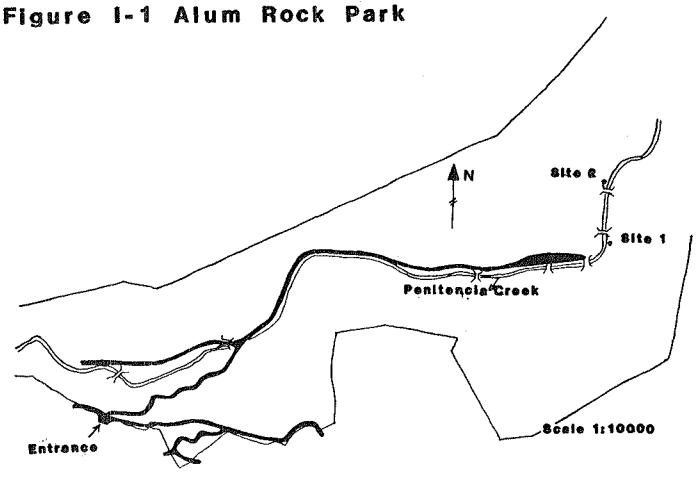
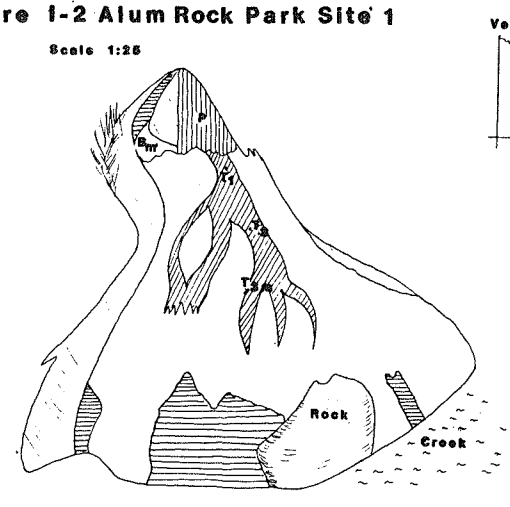
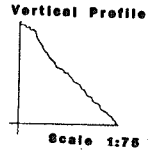


Figure I-2 Alum Rock Park Site 1





Legend

- = Cyanobacteria
- = Purple Bacteria
- = Green Algae
- = White Bacteria

Sample Sites

P = Purple Community
B_m = Cyanobacteria; Motility
T_1 = Thiothrix
T_2 = "
T_{Sm} = " ;*Nostoc*;Motility

portion of the mat consisted of approximately 60 percent *Nostoc*-like filaments (Fig. I-4c) and 40 percent thin *Oscillatoria* (Fig. I-4b). The white part of the mat was made of an almost colorless flexibacterium, possibly *Chloroflexus*. The major portion of the cyanobacterial community, that which was sampled for isotope fractionation studies, was primarily composed of a species of *Oscillatoria* (Fig. I-4a) and coccoids, *Synechococcus* (Fig. I-4e).

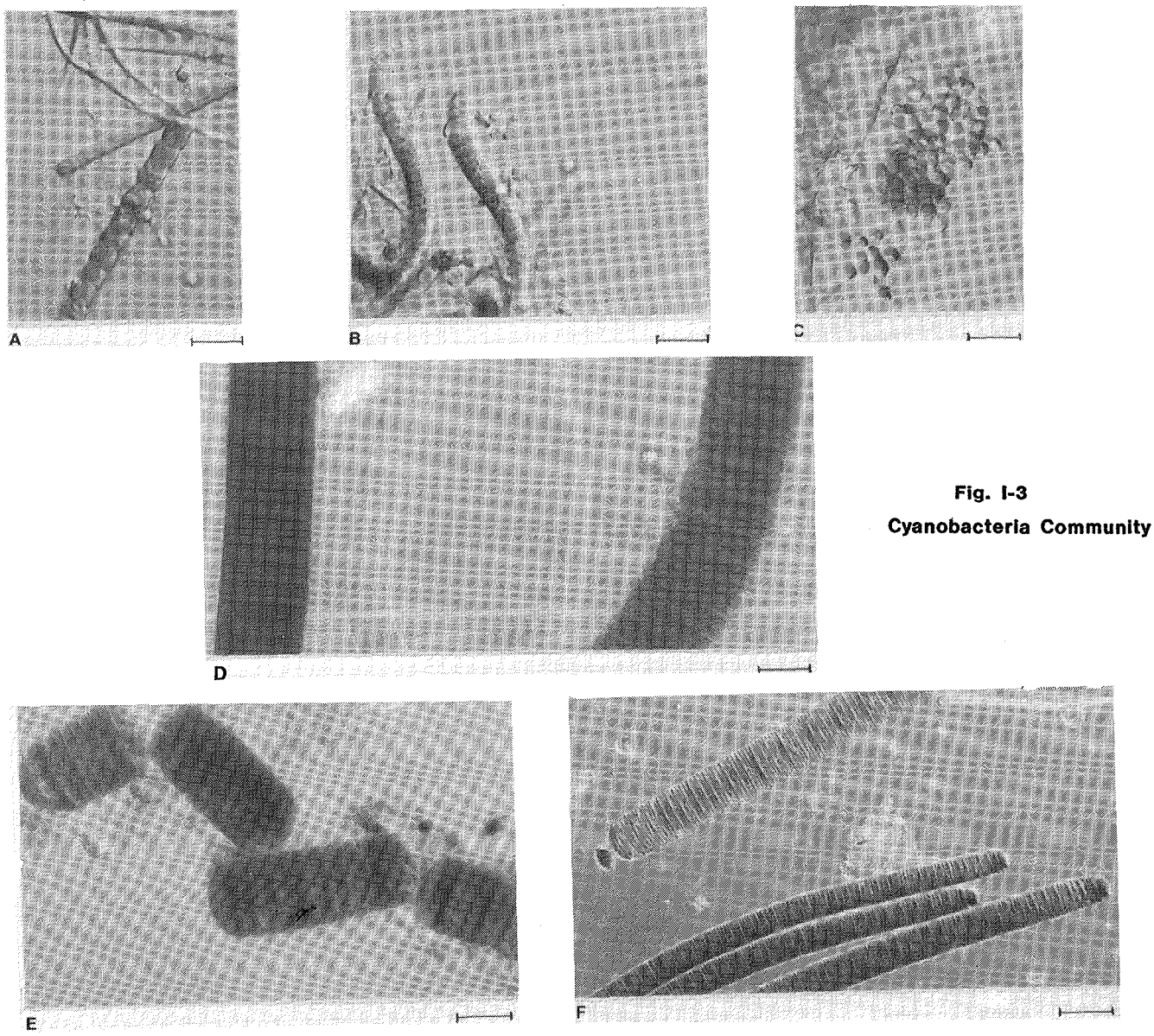
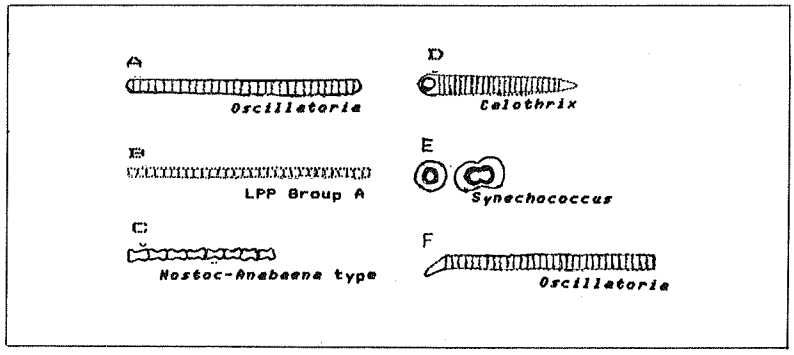


Fig. I-3
Cyanobacteria Community

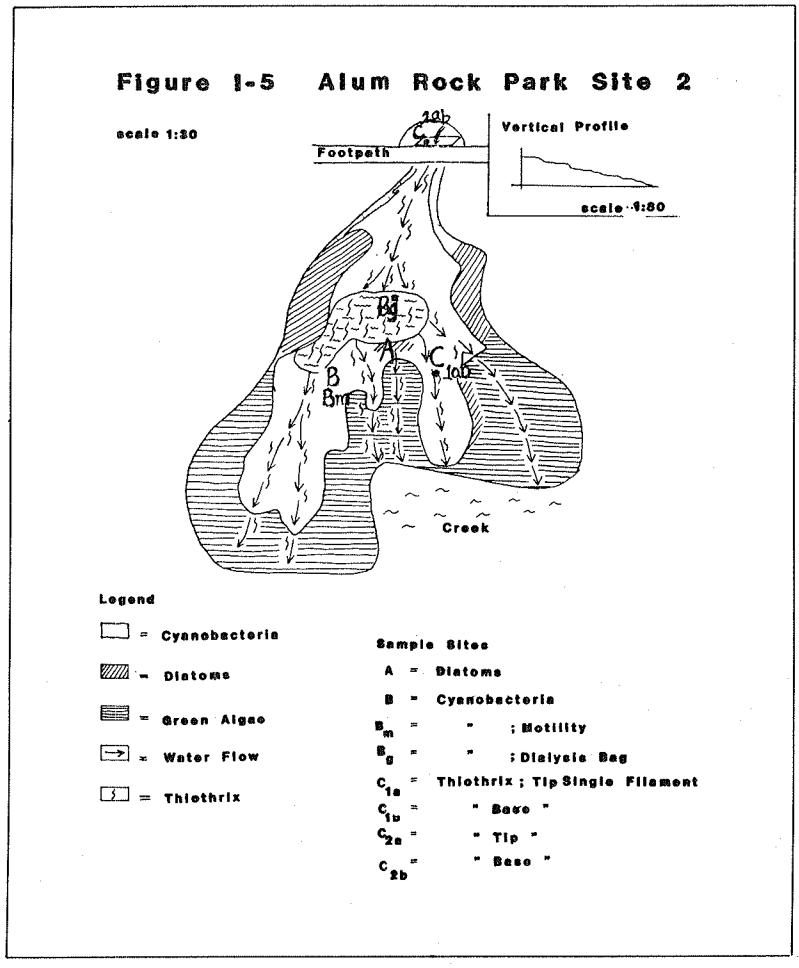
A. Alum Rock Park pillow mat. *Nostoc* sp. Bar equals 15 μ.
B. Alum Rock pillow mat. *Calothrix* sp. Bar equals 15 μ.
C. Alum Rock pillow mat. *Pleurocapsa* sp. Bar equals 15 μ.
D. Salt Marsh *Oscillatoria* sp. (30 μ diameter species) *Beggiatoa* Bar equals 15 μ.
E. Salt Marsh *Oscillatoria hormogonia*. Bar equals 15 μ.
F. Salt Marsh *Oscillatoria* (30 μ diameter and 25 μ diameter species). Bar equals 38 μ.

Fig. I-4 Alum Rock Cyanobacteria



The light green area at the greatest distance from the main water flow consisted mainly of *Spirogyra* with minor quantities of other chlorophytes.

Figure I-5 Alum Rock Park Site 2



Alum Rock Park Site 2 (Figs. I-1 and I-5) is on a gently sloping northern bank of Penitencia Creek. The microbial community at this site was simpler than that at Site 1. The major components of the community were: *Thiothrix*, cyanobacteria, brown-green diatoms and chlorophytes. *Thiothrix*, flapping vigorously, and consisting of a nearly pure population, were attached to the rocks in the main flow stream. The cyanobacterial community consisted nearly exclusively of an *Oscillatoria* distinguished by a hooked tip (Fig. 4f). Within 15 minutes the hooked-tip *Oscillatoria* clumped in culture. Some of the trichomes were identical to hooked tips. The hooked-tip trichomes may have separated from the rest of the trichome by breakage (hormogonia). Since the hooked and non-hooked trichomes persisted through several transfers in liquid media, we considered them to be different morphotypes of the same species.

Palo Alto Baylands Salt Marsh Community

The Palo Alto Baylands salt marsh, a brackish water environment adjacent to San Francisco Bay, was dominated by *Salicornia* and cord grass and underlain by a sulfuretum. At sediment surfaces, especially in less vegetated areas, there were dense mats of microorganisms consisting of surface diatoms, 4-5 types of *Oscillatoria* cyanobacteria or a *Chromatium-Thiocystis* community above sulfate-reducing and methanogenic communities. The *Oscillatoria* community contained at least four different types of *Oscillatoria*, including a dominant gigantic brown-green form (25μm and 30 μm-wide). Cleaned natural samples of this giant *Oscillatoria* were apparently capable of fixing N_2 (see PBME 1980 report). The coexistence of four different morphotypes at one site may indicate different adaptation patterns or ecological advantages, e.g. N_2 fixation, H_2S tolerance, anoxygenic photosynthesis, photoorganotrophy, and so forth.

Alum Rock Park Sulfur Springs

(Kevin Zahnle)

Alum Rock Park, San Jose, California, is the site of sulfur springs containing sulfide and oxidized sulfur compounds as well as a substantial amount of dissolved inorganic carbon. Two sulfur spring sites were selected for study. Site 1 (Figs. I-1, I-2) was a travertine mound below a small 3.3 liter/min spring. The travertine mound indicated that carbonate was actively deposited by the highly CO_2-charged springwater. Dissolved minerals supported dense populations of prokaryotes which covered the mound over its entire two meter height. Site 2 was fed by a fifteen

liter/min spring and was a relatively horizontal spring consisting of several pools and riffles heavily lined with microbial mats and clumps. The odor of volatile sulfides and sulfates was very evident at both sites.

These spring sites were "natural chemostats," which provided nearly steady-state input of water and minerals to communities of prokaryotes. The "chemostats" offered an opportunity to measure the ^{13}C contents of the communities' relatively unmodified environments. The variety of community structures, (microbial mats, clumps and strands bathed in water) offered conditions where both maximal as well as limited expressions of carbon isotopic fractionation could be observed.

To test the assumption that the ^{13}C content of the springwater was nearly constant, water samples were analyzed both upstream and downstream of the sites where microorganisms were sampled. As Tables I-I and I-III indicate, the *delta* ^{13}C of the total dissolved carbon increased downstream along the spring runs (compare the "top in" with "bottom out" values.) These *delta* ^{13}C increases (about 2.2 $^{o}/_{oo}$ for Site 1 and less than 1.0 $^{o}/_{oo}$ for Site 2) reflected the outgassing of relatively ^{13}C-depleted CO_2 from the waters. The greater *delta* ^{13}C shift observed for Site 1

Table I-I Isotope Fractionation Data for Bacteria Isolated from Alum Rock Park, Site 1

Water[a]	Temp.	Eh	pH	CO_2 (content)	delta $^{13}CO_2$ (total) (parts per thousand)	delta $^{13}CO_2$ (parts per thousand)[2]	Apparent Fractionation (average) delta ^{13}C (parts per thousand)
top in	28.6°C	} -310	6.43	} 48 mM/l	- 6.5±0.2[d]	-10.5	----
top in	28.6		6.58		- 6.5	-11.0	----
bottom out	27.8	} -265	6.87	} 36 mM/l	- 4.3	- 9.8	----
bottom out	27.8		7.20		- 4.3	-10.9	----
Organisms							
purple (+ green sulfur) bacteria at top (largely Chromatium sp. and Chlorobium sp.)					-27.9[e]	----	-17.7
a) Thiothrix spp. at top					-16.5		- 5.8
b) Cyanobacteria and community at top					-12.1		- 1.3
c) Motility selected cyanobacteria at top					-28.4		-17.7
d) Motility selected cyanobacteria at bottom					-26.5	----	-16.1
e) Thiothrix at bottom (and bluegreen contamination)					-29.0		-19.6

[a]Water flow was 3.3 liters/minute.

[b]Errors are for mass spec measurements alone. They are doubtless small compared to other, less easily quantified errors that could have arisen anyplace between site and datum.

[c]delta $^{13}CO_2$ refers to dissolved CO_2. It is calculated assuming isotopic equilibrium between CO_2 (aq) and HCO_3^-. This may not be a wholly justified assumption.

[d]Total carbonate as CO_2.

[e]Cell mass as CO_2.

was consistent with the greater CO_2 loss, which caused rapid carbonate deposition. This rapid deposition was evidenced by the travertine mound and by the greater decrease in dissolved inorganic carbon observed across that site (Table I-I.) Fortunately these *delta* ^{13}C increases were not so large as to obscure the biological isotopic fractionation which was the object of this study.

Table I-II Isotope Fractionation Data for Diatoms and Bacteria Isolated from Alum Rock Park, Site 2

Water[a]	Temp.	Eh	pH	CO_2 (content)	delta $^{13}CO_2$ (total) (parts per thousand)	delta $^{13}CO_2$ (parts per thousand)	Apparent Fractionation (average) delta ^{13}C (parts per thousand)
in	} 27.6°C	} -231	7.42	} 36 mM/l	- 4.8	-11.9	
in			7.37		- 4.0	-11.1	
out	} 27.0°C	} -187	7.49	} 28 mM/l	- 3.6	-10.7	
out			7.65		- 4.0	-11.1	
organisms							
diatoms					-17.3		- 6.1
Bluegreen community				LOST			
Bluegreen motility selected					-23.7		-12.5
Oscillatoria spp. purified and returned to field for 48 hours.					-18.5		
Oscillatoria spp. purified and returned to field for 48 hours.					-19.3		- 7.7
Thiothrix							
set 1 { overall					-34.4		
set 1 { overall					-34.4 }		-22.9
set 1 { overall					-33.1		
set 2 { tips at top					-32.6 }		-21.5
set 2 { tips at top					-32.9		
set 2 { clumps at top					-30.7 }		-19.2
set 2 { clumps at top					-30.1		
set 2 { tips at bottom					-31.8		-20.6
set 2 { bases at bottom					-32.2		-21.0

[a] Flow rate = 15 l min^{-1}

"Apparent Fractionation" values (Table I-I) were obtained from purple and green phototrophic bacteria which grew chiefly near the spring outlets, and from cyanobacteria and chemolithotrophic bacteria, primarily *Thiothrix* which grew in abundance all along the spring runs. These isotope values, which are discussed below, denote the difference between the *delta* ^{13}C values of the organisms and the *delta* ^{13}C value of their inorganic carbon source, assumed here to be aqueous CO_2 in equilibrium with other aqueous carbon compounds at the temperature and pH indicated in the table.

A substantial fractionation (-17.7 $^{o}/_{oo}$) was observed for the purple and green phototrophic bacteria, which grew at the top of the travertine mound. This fractionation was consistent with values obtained for *Chromatium* in the laboratory (Quandt *et al.*, 1977; Sirevag *et al.*, 1977). Even though these colonies grew as mats and clumps, the

substantial fractionation indicates that, due to the high inorganic carbon content of the water and/or the slow growth rate of these bacteria, the rate of carbon dioxide supply during their growth exceeded the rate of enzymatic uptake of carbon. Also the large fractionation suggests that purple, rather than green sulfur bacteria were principally responsible for this carbon fixation because even impure cultures of green sulfur bacteria do not discriminate against ^{13}C to this extent (Sirevag *et al.*, 1977; Quandt *et al.*, 1977).

Both the cyanobacterial and the *Thiothrix* communities exhibited low isotopic fractionation. Although such a low fractionation has been observed in cyanobacterial mats (Barghoorn *et al.*, 1977) the apparent fractionation for *Thiothrix* here was much less than all values reported for it elsewhere in this work (see Tables I-I and I-II.) Although such low

Table I-III Isotope Fractionation Data for Bacteria Isolated from the Palo Alto Baylands Salt Marsh

Water	Temp.	Eh	pH	CO_2 (content)	delta $^{13}CO_2$ (total) (parts per thousand)	delta $^{13}CO_2$ (parts per thousand)	Apparent Fractionation (average) delta ^{13}C (parts per thousand)
pond	24°C	+100	8.1	2 mM/1 (3.6)[a]	-10.2[d]	-18.1	
black sediment	----	-400	7.2	----			
mat	----	-380	6.82	----	----	-16.6	
pond	----	+100	8.1	2 mM/1	-10.6	-18.5	
Organisms							
purple phototrophic bacteria (Chromatium sp. and Thiocystis sp.)					-21.5[e]		- 3.2
purple phototrophic bacteria (Chromatium sp. and Thiocystis sp.)					-21.6		
dark bluegreen dominated mat, 24 µm (contains 30 µ Oscillatoria, 7 µm Oscillatoria, diatoms, nematode worms, fecal matter, a few Lpp forms)					-18.4 -18.4		- 0.1
motility selected cyanobacteria					-24.1		- 5.8
purple prototrophs by culture[b]:							
Chromatium sp.					-56.1		-17.2
Thiocystis sp.					-56.4		-17.5
Mentum[c]				22.4 mM/1	-33.1	-38.9	----

[a] Methanogen group supplied water.

[b] Phototroph group supplied bacteria.

[c] Phototroph group supplied their results: J. Chanto K. Kneller.

[d] Total carbonate as CO_2.

[e] Cell mass as CO_2.

fractionation suggested that the rate of enzymatic carbon assimilation exceeded the rate of CO_2 supply, perhaps the ^{13}C-enrichment was also enhanced by the isotopically heavy carbonate precipitating among the cells at the top of the mound. Carbonate was being deposited at the top and its precipitation rate would have been enhanced among actively growing cells where CO_2 uptake is occurring. Note that the *Thiothrix* harvested at the bottom of the mound (Table I-I) was relatively

^{13}C depleted, reflecting the absence of significant carbonate precipitation. The significant fractionation value (*delta* ^{13}C = -29 $^{o}/_{oo}$) for *Thiothrix* at the bottom is consistent with the observation that its enhanced fractionation may reflect a greater dependence of the community on autotrophic nutrition. This organism contains RuBP carboxylase.

Isotopic fractionation was measured in cyanobacteria which glided into clumps of wet quartz wool placed on top of the mats. This technique mainly selected for *Oscillatoria* which can glide by phototaxis and therefore concentrate themselves into a dense, nearly pure population. These cyanobacteria travelled 1 to 2 cm in 24 to 48 hours and occupied the quartz wool. As Table I-I shows, these "motility selected" cyanobacteria exhibited fractionated ^{12}C to a significantly greater extent than the mixed community comprising the cyanobacterial mat. This fractionation probably reflects a greater rate of CO_2 supply to the cells relative to the rate of RuBP carboxylase activity. Two reasons may have accounted for a greater rate of CO_2 supply: lower cell densities in the wool and therefore less competition for CO_2, or the absence of cyanobacteria sheath material surrounding these cells. Sheaths surrounding the cells may have reduced the rate of CO_2 transported to the cells.*

Alum Rock Park Site 2 had a larger stream with a lower gradient and bigger pools than did Site 1. The experiments conducted at Site 2 were as follows:

Water and microbial community samples were taken as at Site 1

Oscillatoria were sampled and purified and returned to the stream in a dialysis bag for 48 hours, and

Clumps and free-floating filaments were analyzed from *Thiothrix*, an organism for which isotopic analyses of almost pure natural enrichment cultures have not previously been reported.

The apparent fractionation for the *Oscillatoria* in the dialysis bag experiment was *delta* ^{13}C = -7.7 $^{o}/_{oo}$, which exceeds the fractionation observed for cyanobacteria at Site 1. Unfortunately the cyanobacteria mat sample from Site 2 was lost during analysis. Also, the *delta* ^{13}C value of the cells at the beginning of the 48 hour growth period in the dialysis bag was unfortunately not measured.

Cyanobacteria were again selected for motility, and as was seen at Site 1, the motile cells displayed a larger isotopic fractionation than was observed for other cyanobacterial cells at

* See Appendix V on interpretation of carbon isotope values.

the site.

The analyses of *Thiothrix* were performed in two sets (Table I-II.) Reconnaissance measurements were performed early in the study (set 1) with randomly selected samples. These samples were mostly the middles and tips of *Thiothrix* trichomes dangling in free-flowing water. Isotope results obtained from samples taken at different times were in agreement. The set 2 samples were carefully selected trichome "tips," "bases" (attachment sites where water flow was somewhat restricted) and "clumps" (clumped strands within which water flow was more restricted than in the "base" samples). At the upstream end of Site 2, the *Thiothrix* "clumps" were enriched in ^{13}C by about 2 $^{o}/_{oo}$, relative to the "tips". This difference may have been due to a slower rate of CO_2 delivery in the "clumps". There was no apparent difference between "tips" and "bases" downstream, suggesting that the rate of carbon delivery to the "base" cells exceeded the delivery rate in the "clumps." Note that the carbon fractionation by *Thiothrix* exceeded that of any other organism studied at this site by this group. This fractionation was consistent with the presence of RuBP carboxylase, the rate of carbon fixation of which does not equal or exceed the rate of inorganic carbon supply. The growth rate of *Thiothrix* may have been limited by the availability of sulfide, a factor which would have attenuated the carbon fixation rate.

Palo Alto Baylands Salt Marsh Community

The marsh site, near the Baylands Nature Interpretive Center on Embarcadero Road in Palo Alto, is a tidal marsh with flat stagnant pools and black muds populated both by grass and by mats of bacteria and chlorophytes. Cyanobacteria and phototrophic bacteria were easily sampled. Phototrophic bacteria were also obtained from the PBME Phototrophic Bacteria Group, which enriched and isolated organisms taken from this salt marsh (see Chapter II).

As Table I-III shows, the salt marsh pond water contained much less dissolved carbon than the Alum Rock water (less than one tenth). The *delta* ^{13}C value of this carbon was also markedly lower than that of seawater bicarbonate (typically 0 $^{o}/_{oo}$). Perhaps ^{13}C-depleted carbon dioxide, produced by the breakdown of organic matter in the mud by fermentation and sulfate reduction was entering the pond water.

The purple phototrophic bacteria and cyanobacteria in the

marsh showed little or no apparent isotopic fractionation, which may have reflected competition for limited quantities of inorganic carbon. If competition was causing the low apparent fractionation, the remaining inorganic carbon in the mats should have been markedly enriched in ^{13}C. But neither the isotopic composition of the mat nor of the sediment water could be measured due to time limitations.

Pure cultures of *Chromatium* and *Thiocystis* obtained from the salt marsh were analyzed isotopically. As Table I-III indicates, these organisms produced much larger isotopic fractionation in the laboratory cultures than they did in the marsh. This difference suggests that the rate of carbon supply exceeded the rate of carbon fixation by a wider margin in the laboratory cultures than it did in the marsh. That the inorganic carbon concentration in the culture medium exceeded the marsh pond water by more than tenfold (Table I-III) could easily have accounted for this observed difference in fractionation.

Moist quartz wool was placed in the marsh at Baylands Nature Center to obtain enriched samples of motile cyanobacteria. These *Oscillatoria* were more ^{13}C-depleted than were the mat *Oscillatoria*, suggesting either decreased competition for carbon and/or enhanced delivery rate of carbon due to the absence of sheaths in the quartz wool colony. Note, however, that the apparent fractionation for these motile *Oscillatoria* was less than that observed for the motile *Oscillatoria* at Alum Rock Park (Table I-III vs. Tables I-I and I-II). This lower fractionation may have been due to the lower concentration of inorganic carbon in the water. At low extracellular carbon concentrations, cyanobacteria concentrate inorganic carbon in their cells at levels greatly exceeding extracellular inorganic carbon (Kaplan *et al.*, 1980). This concentration mechanism which inhibits exchange of carbon between the cell and its environment leads to lower apparent isotopic fractionation.

Large pieces of glass wool placed above the mat surface in a cylinder forced the motile bacterial components of the mat to move upward phototactically. New growth occurred toward the new upper surface. The thickness of each of the mat layers expanded, while the stratigraphic position of the layers appeared to remain constant. (Experimentally induced expansion of the mat layers also has been seen in salt microbial mats of the Sabkha Gavish, Israel by Friedmann and Krumbein, in press.) The mat layers expanded from about 0.1 mm to 1 mm, or from 10 to 100 times, due to enhanced transmission of light through salt crystals or through quartz wool fibers which were very translucent. The expansion may also have allowed greater exchange for CO_2 into the layers.

Time limitations precluded gathering and storing samples for microscopy, chemistry and isotopic analysis simultaneously. A map of the mat site was drawn. Within the five week study the community changed occasionally. Water from within the mats, at the level from which cells were taken, was studied. Since the community was patchy on a sub-millimeter scale, microsampling and microelectrode techniques were correlated with identifications.

ISOLATION OF CYANOBACTERIA AND DESCRIPTION OF CULTURES

(Carmen Aguilar-Diaz)

Media

Using the different media listed below, (pH 7.0-8.3) we tried to isolate the different types of cyanobacteria from their natural environments.

BG 11 and BG 11° for the freshwater strains

ASN III and ASN III° for the marine strains (Rippka *et al.*, 1979)

Medium D (pH 8.3) for thermophilic strains (Castenholz, 1981), used for Alum Rock Park cultures.

We used the media in several ways: liquid, pour plates, agar shakes, enrichment of the atmosphere with CO_2 (in the case of the liquid media), and addition of H_2 or selenium (see Appendix I). In the case of the agar, we used Difco Bacto-Agar (final concentration 1 percent (w/v Rippka *et al.*, 1979). The light source was cold fluorescent white lamps.

Isolation

We also used different isolation techniques:

Single filament transfer

In the first technique we cleaned the fresh samples with filter-sterilized water (Alum Rock Park and salt marsh water). Then we picked out single filaments with a probe and transferred them to an agar plate with the aid of a dissecting microscope. After 2 days or so, we transferred them to fresh plates, and incubated them at room temperature.

Isolation of motile trichomes

In the second technique inoculation of cyanobacteria in the center of an agar plate allowed the gliding trichomes to move out from the inoculum, making it easy to transfer the isolated ones. The mucus trails left behind tended to get contaminated with heterotrophic bacteria. After trichome migration, we cut the piece of agar in which they were embedded and placed it on a new agar surface. Most filaments were motile in every phase of growth. We also isolated the trichomes of cyanobacteria by placing several glass slides in a vertical postion into the sample. After twelve hours a large number of filaments clean enough to use for the first inoculum glided up the slide.

Manual isolations with the aid of a dissecting microscope

In another isolation technique we washed masses of recognizable filaments with sterile water or medium. We inoculated them by means of a loop, by submerging them in the agar, or just by placing the inoculum into the sterile liquid medium.

Agar shakes and dilution series

In the last technique we used a small inoculum placed in melted agar (T=45° C) and then homogenized with a vortex. Once the agar had solidified, it was incubated at room temperature and exposed to continuous light. The dilutions, from 10^{-1} to 10^{-6}, were poured onto plates afterwards.

Purification

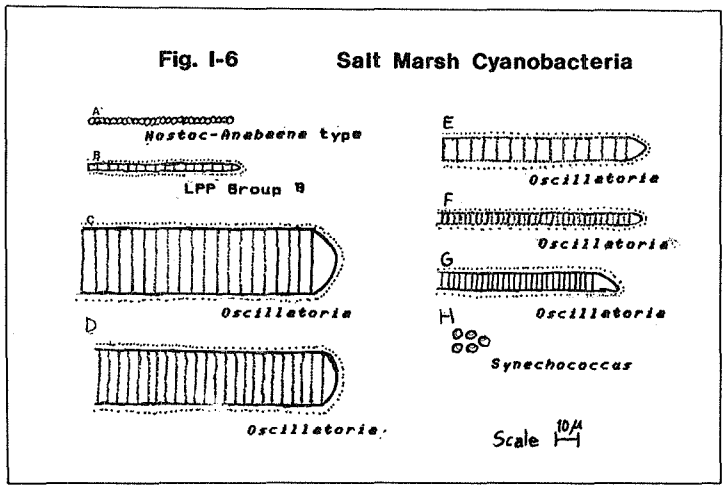
To purify the cleanest cultures, we transferred pieces of agar containing filaments into cyclohexamide (10^{-4} *M* final concentration) to rid the culture of the accompanying eukaryotic organisms.

Results

The general appearance of the Alum Rock Park and salt marsh organisms is shown in Fig. I-3. In general, we observed growth in the liquid freshwater medium (BG 11) both in the Alum Rock Park and in the salt marsh samples. Most salt marsh strains did not grow when transferred directly from field samples into the liquid medium (ASN III). They grew better in pour plates of that medium, and we transferred them several times in it before we finally placed them in the sterile ASN III liquid medium. The

use of BG 11° and ASN III° was not very successful. During the microscopic observations of the samples of the two environments, we only found two heterocystous cyanobacteria (*Calothrix* Fig. I-4d) in the Alum Rock Park samples. *Nostoc* was overgrown in culture by an *Oscillatoria* sp. (Fig. I-4f) and eventually died. In the Alum Rock Park liquid BG 11 medium we obtained a monocyanobacterial culture of the *Oscillatoria* type (Fig. I-4f) and a mixed one containing two *Oscillatoria*-type trichomes (Fig. I-4a and I-4f). To purify these cultures, we added cyclohexamide (10^{-4} *M*) to inhibit the growth of eukaryotic organisms and transferred the cultures. Small amounts of the culture (0.5 percent) were transfered to new sterile liquid medium. The oscillatorians were clean but not axenic.

We obtained the large *Oscillatoria* (Fig. I-6c) on BG 11 liquid medium from salt marsh samples in almost pure culture for several weeks. Two types of oscillatorians (Fig. I-6c and Fig. I-6d) and one LPP group B (Fig. I-6b) continued to grow in mixed culture until the end of the course.



A poorly growing mixed culture of coccoids (Fig. I-6h) and oscillatorians (Fig. I-6f) and one monocyanobacterial culture of the *Oscillatoria* type (Fig. I-6e) were enriched in ASN III medium.

We obtained a mixed culture of *Nostoc-Anabaena* type (Fig. I-4a) and *Oscillatoria* type (Fig. I-4e), using liquid medium D for the Alum Rock Park samples as suggested by Dr. B. Pierson. This medium was useful for the cultivation of cyanobacteria found at temperatures lower than 30° C. It also supported good growth of a salt marsh *Oscillatoria* sp. (Fig. I-6e) and some coccoids (Fig. I-6h).

The agar plate cultures showed good growth of cyanobacteria from both environments. The dominant form on BG 11 from Alum Rock Park was an *Oscillatoria* type (Fig. I-4e). We obtained the growth of two salt marsh oscillatorian types, one with a 26 μm width (Fig. I-6d) and one with a 5.2 μm width (Fig. I-6f), as well as thin filaments, on ASN III medium. In the agar shakes we observed the healthy growth of an LPP-B cyanobacterium in both media, most of which were mixed cultures. However different *Oscillatoria* type cyanobacteria grew uncontaminated, though slowly, in ASN III agar medium (Fig. I-6). Since we observed growth of the 5.2 μm and the 7.8 μm oscillatorians in the anaerobic area of the tube, we transferred them for purification to see if they grew anaerobically on sulfide.

The isolation techniques for the various cyanobacteria were useful. We varied the conditions by using different H_2S concentrations (1,2,3,4 and 5 μM; Padan, 1978) and NH_4Cl (0.01, 0.02 and 0.04 M) instead of the usual nitrogen source (NO_3) in the medium growing the larger *Oscillatorians* (25-30 μM). But the experiments were not continued long enough to obtain results.

Cyanobacteria are very difficult to isolate and purify in a short interval of time since they do not grow fast. With this in mind, we think that the results of our work during only six weeks were amazing. At the end, cultures were growing in good health.

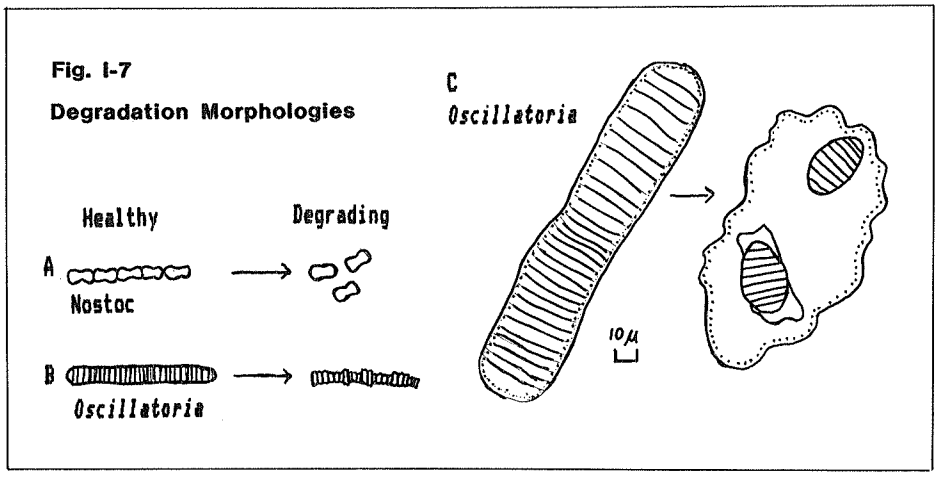
We took the isolated material to our home labs to be purified. The work is continuing there: Geomicrobiology, University of Oldenburg, Oldenburg, West Germany; Geology, Louisiana State Univeristy, Baton Rouge, LA; and Laboratory of Bacteriology, Mexico City, Mexico.

Description of Cultures

Morphologies of degrading cyanobacteria

In the prePhanerozoic fossil record, cyanobacteria-like organisms have been described, and in many cases classified on the basis of their morphology. But it is important to remember that the morphology of unhealthy cells and degrading cells may differ significantly from their healthy counterparts. Some examples of this were observed during microscopic study of field samples and cultures. The Alum Rock Park *Nostoc* degraded into single pinched cells that could easily have been mistaken for dividing coccoids (Fig. I-7a). Many of the *Oscillatoria*

species when dying or dead exhibited differential shrinkage of cells that left intact some large cells reminiscent of heterocysts, the nitrogen-fixing cells whose presence is one basis for generic distinctions (Fig. I-7b). The two large salt marsh *Oscillatoria* actually exploded while being viewed under the microscope, presumably because of osmotic pressure changes. The result was a cloud of extruded intracellular material containing nearly spherical packages of cells (Fig. I-7c).



These observations support the contention of many paleobiologists that classification of microfossils according to the morphology preserved in the rock record is risky at best.

Summary

Four different strains of cyanobacteria from Alum Rock Park were cultured. These include *Oscillatoria* type, *Nostoc/Anabaena* type, and *Calothrix*. From the salt marsh five *Oscillatoria*-type cyanobacteria, one *Nostoc/Anabaena* type, one LPP-B type, and one coccoid type were isolated. All the different media we used were useful, but BG 11 and D media seemed to be the most versatile. The use of selenium did not seem to benefit or impede growth, as determined from the inoculations into media with and without selenium.

CHEMICAL ANALYSIS OF MICROBIAL MATS OF ALUM ROCK PARK

(Margriet Nip)

In order to compare measurements of chlorophyll *a* and protein contents per square centimeter with microscopic investigations and field site isotope fractionation studies,

three samples from Site 1 and two samples from Site 2 of Alum Rock Park were analyzed. (For methodology, see Krumbein *et al.*, 1977, Jorgensen and Cohen,1977). Total protein content was determined by the method of Lowry, *et al.*, 1951.

The results are shown in Table I-IV. The chlorophyll *a*-protein ratios of the field samples seem to correlate quite well with the microscopic investigation of the samples with respect to the relative abundances of the different organisms. Those samples dominated by photosynthetic oxygenic organisms (approximately 80 percent) displayed chlorophyll *a* to protein ratios between 1:100 and 1:200. Those samples dominated by sulfur oxidizing bacteria showed much lower chlorophyll *a* to protein ratios (about 1:7000).

Table I-IV Content of Chlorophyll a and Total Protein in Species of Cyanobacteria and Diatoms

Sample #	Sample Site	Sample Material	Chlorophyll a Content ($\mu g/cm^2$)	Protein Content (mg/cm^2)	Chlorophyll a: Protein
1	T_{3m}	Flexibacteria with a thin layer of cyanobacteria (60% LPP Group B, 40% Oscillatoria), ∿ 80% cyanobacteria	251.0	43.6	1: 173.71
2	P	Chromatium mixed with Thiothrix and Chlorobium, ∿70-75% Chromatia and Chlorobium	30.336	27.0	1: 890.03
3	T_1	Thiothrix with Oscillatoria sp. and few Anabaena, > 95% Thiothrix	12.616	88.0	1:6975.27
4	B/B_m	Thiothrix with a thin layer of Oscillatoria sp.; approximately 70% Oscillatoria, 30% Thiothrix	29.8	48.0	1:1610.74
5	A	Diatom, > 80% diatoms	338.26	37.6	1: 111.16

Since the chlorophyll *a* and protein measurements were consistent with the microscopic investigation on the samples, we concluded that the carbon isotope fractionation data described in the following section can be regarded as being representative for communities dominated by different organisms. All the different field samples showed different patterns of *delta* ^{13}C fractionation, although many of the organisms in them are known to use the RuBP carboxylase pathway for CO_2 fixation. Some of the obligatory anoxygenic photosynthetic bacteria used a reverse TCA-cycle and fixed CO_2 in different ways (Sirevag *et al.*, 1979).

ISOTOPE FIELD EXPERIMENTS: NATURAL CHEMOSTAT

(Carmen Aguilar-Diaz, Kent Sprague, Kevin Zahnle)

Three sites were selected for studies of community isotope fractionation. The two sites of Alum Rock Park were selected because they act like "natural chemostats" providing nearly steady-state input of water and minerals to the specified community. The water flowing through the community had an almost neutral pH, and was very high in sulfide and oxidized sulfur compounds, as well as having had a high total of dissolved CO_2/HCO_3^-. Area limitations of the communities were designated not by natural boundaries, but by the need for consistency among the workers of our group, and for the occurrence of organisms of interest to us. Once the field designations were assigned, as shown on the sketch maps, they were retained through the project (Figs. I-1,I-2,I-5). The salt marsh site was chosen for accessibility and the presence of extensive microbial mat cover.

The object of the field studies was to evaluate different isotopic fractionation in the communities of horizontally, gradually changing and stratified microbial mats. Samples taken from the field often had a large concentration of the species of interest, and were therefore considered isotopically identical to the species itself. A picture of how organisms fractionate relative to each other under natural conditions in the presence of excess sulfide and bicarbonate could be estimated using our data (Tables I-I, I-II, I-III). The use of "natural chemostats" allowed us to look for maximal fractionation and cases of closed system effects, as when the organisms were clumped in a mat. In the cyanobacteria motility studies, quartz wool was placed on top of the dark green patches. Some of the species of cyanobacteria (mainly *Oscillatoria*) glided up by phototropotaxis seeking available surfaces. The cyanobacteria with few others concentrated themselves. The cyanobacteria travelled through 1-2 cm and "filled" the quartz wool in 24-48 hours. The hypothesis was that they would be isotopically heavier because of their greater access to CO_2 in the air and their exchange of it to reach equilibrium due to the continuous flushing of water in the wool.

Our first set of total CO_2 and isotope analyses was performed in order to evaluate the suitability of the Alum Rock Park sites for community studies. The incoming and outgoing water was tested in order to determine whether there were excessive kinetic effects which might mask biogenic isotope fractionation and *delta* ^{13}C fractionation between HCO_3^- and CO_2 caused by diffusion. Since there was almost no difference in *delta* ^{13}C between ingoing

effects could be neglected in Sites 1 and 2 of Alum Rock. Biologic fractionation upstream could be neglected as well because of the high rate of water flow and dissolved carbonate relative to the biomass of bacteria.

Alum Rock Park Site 1

Upstream and downstream, various organisms from the steep community were sampled and total cellular carbon was analyzed. The upstream sample was composed of purple and green phototrophic sulfur bacteria. The carbon isotope fractionation of ^{13}C was approximately -16 $^{o}/_{oo}$, which was expected. *Thiothrix* was sampled and analyzed, but the value of this datum (-5.8) is questionable because of its disagreement with the other seven determinations on this organism. (The fractionation ranged from -19.2 to -22.9) We also obtained an anomalous *delta* ^{13}C number for the cyanobacteria living in the top inlet (-1.3). These very isotopically heavy values could indicate a mat with atmospheric exchange effects (limiting CO_2), or the presence of contamination by travertine granules or C_4 plant carbon.

The cyanobacteria selected by their motility were enriched in ^{12}C indicating RuBP carboxylase CO_2 fixation in excess CO_2. The motility-selected cyanobacteria moved quickly away from most other organisms onto the quartz wool placed on top of the mat. Their isotopically light values (-28.4 to -26.5) suggest they grew under conditions favoring fractionaton.

Thiothrix mixed with cyanobacteria within the clump was obtained near the outlet. The isotope fractionation value (-29.0) was intermediate between the expected values for cyanobacteria or *Thiothrix* alone.

Alum Rock Site 2

Site 2 is a flat community with some standing pools of water. The experiments for this community were:

Analysis of the community structure, as at Site 1;

Obtaining isotope values for *Thiothrix*, since these have not previously been reported for almost pure natural enrichments of these organisms;

Placing *Oscillatoria*, taken into culture and purified from this site, back in the environment for 48 hours in a dialysis bag.

The diatoms, showing relatively light ^{13}C values (-17.1) may have been CO_2 limited. The cyanobacteria selected by motility (*delta* ^{13}C = -23.7 $^{o}/_{oo}$) including those purified and returned to the field also showed large fractionation values. The cyanobacteria from both sites fractionate in their natural setting within the normal range expected from published data (Pardue, *et al.*, 1975). The small (-7.7 $^{o}/_{oo}$) fractionation value by the *Oscillatoria* in the dialysis bag was not certain because the *delta* ^{13}C for the cells at the beginning of the experiment was not measured.

The *Thiothrix* measurements were done in two stages. Survey measurements were performed early in the study with randomly selected samples (probably mostly middle parts and tips of strands dangling in the free-flowing water). These results were consistent at different sampling times. *Thiothrix*, relative to other community members, showed consistently isotopically light values. These chemolithotrophic bacteria, bathed constantly in CO_2-rich water, preferentially used the isotopically light CO_2. The second set of samples (set 2, Table I-I) were selected for trichome "tips" (the bacteria at the end of a long strand bathed in rapidly flowing water), "bases" (attachment sites where clumping may cause some limitation of CO_2) and "clumps" (free-flowing clumped strands inside of which limitation of CO_2 might occur). Large apparent fractionation was consistently estimated (from -19 to -23 $^{o}/_{oo}$). A major source of isotope fractionation in sedimentary carbon may be due to that performed by sulfide oxidizing autotrophs. There was no indication of CO_2 limitation for *Thiothrix* at the bottom of Site 2. All *Thiothrix* samples showed *delta* ^{13}C values between -30.1 and -34.4.

Salt Marsh Community

The data for this study are in two parts. We studied the field community of microbial mats found in the marsh, and almost pure cultures of purple sulfur bacteria obtained from the Trueper lab group.

Purple sulfur phototrophic bacteria and some mat cyanobacteria showed little or no apparent fractionation under natural conditions in the field. A relatively homogeneous field sample consisting of mostly *Chromatium* and *Thiocystis* was sampled and the *delta* ^{13}C determined. The apparent fractionation was only -3.2 $^{o}/_{oo}$ *delta* ^{13}C. The CO_2 in low concentrations in the water around the mat

was isotopically light (-10 $^{o}/_{oo}$), relative to the Alum Rock Park water (-4 $^{o}/_{oo}$).

Motile cyanobacteria did fractionate significantly, becoming lighter as they exchanged CO_2 with air in the quartz wool. It would be interesting to grow some of the *Oscillatoria* in pure culture in the lab under high and very low CO_2 concentrations for 1-2 weeks, and then place them out in the salt marsh for 4 or 5 days on quartz wool (on floats to stay at the top of the water). The high CO_2 culture might be expected to fractionate to a greater extent than the low CO_2 culture.

Almost pure cultures of both organisms isolated from the salt marsh were obtained from D. Craven and *delta* ^{13}C assayed. In both cases the *delta* ^{13}C was very light compared to the orginal *delta* ^{13}C signature of CO_2 in the medium. Thus the capability of cultured phototrophic bacteria to select ^{12}C is firmly established. This information reinforces the conclusion that the salt marsh mat environment was CO_2 limited. The phototrophic bacteria were forced either to use more of the ^{13}C-rich CO_2 under those natural conditions, or to depend more on heterotrophy.

CARBON ISOTOPE FRACTIONATION BY *PHORMIDIUM*: LABORATORY STUDIES

(Carmen Aguilar-Diaz, Kent Sprague and Kevin Zahnle)

We investigated carbon isotope fractionation by *Phormidium luridium* in a variety of media (data summarized in Table I-V). The basic stock medium was BG 11, but the carbonate buffer was omitted in order to better control the isotopic composition of CO_2. The cultures were gassed for two minutes every six hours with the given gas composition. They were grown for 72 hours (with the exception of culture 1, which was grown for 48 hours). The CO_2 used for gassing was isotopically light (*delta* ^{13}C = -38.9 $^{o}/_{oo}$). DCMU was added to some media in order to block Photosystem II, preventing the use of water as electron donor in photosynthesis. Isotopically heavy glucose (*delta* ^{13}C = -9 $^{o}/_{oo}$) was added to some media. If the glucose were incorporated into cells, the cells would become isotopically heavier. Medium 5 was particularly interesting, as here we tested for the ability of *Phormidium* to use glucose as an electron donor in photosynthesis.

The method of discontinuous gassing was inferior to continuous gassing. It permitted oscillation in the *delta* ^{13}C due to the closed system effect. However, the gassing was rapid enough compared to the culture's growth rates that we expected no problem to arise. We therefore concluded that the organisms used CO_2 at *delta* $^{13}C = -38.9\ ^{o}/_{oo}$, because this was the input reservoir. This predicted that the media samples which were dominated by the isotopically heavy bicarbonate would give an isotopic ratio of *delta* $^{13}C = -32.2\ ^{o}/_{oo}$. Our experimental results (Table I-V) disagreed rather strongly with this prediction. It is unclear which (if either) of these

Table I-V **Photo- (Organo-) and Heterotrophy Experiment Using Pure Cultures of *Phormidium luridium***

Designation/ Description	Potential Energy Source(s)	Potential Electron Donor(s)	Potential Carbon Source(s)	O_2	Relative Cell Densities (arbitrary units)	pH	delta ^{13}C for Cell Material, parts per thousand	delta ^{13}C for Medium, parts per thousand HCO_3^-	CO_2	mM CO_2/l* Total CO_2 in Medium	Relative Fractionation[e] delta ^{13}C, parts per thousand
1a BG11[a] + air +	light	H_2O	CO_2	+	0.45	6.8-	-----	-18.2	-26.2	3.5	
1b 5% CO_2[b]				+	0.45	7.3	-53.4±0.2	-22.6	-30.6	2.7	-14.5, -22.8
2a BG11 + N_2 +	light	H_2O	CO_2	-	0.58	6.8-	-54.4±0.2	-21.5	-29.5		-15.5, -24.9
2b 5% CO_2				-	0.58	7.3	-55.7±0.2	-22.8	-30.8		-16.8, -24.9
3a BG11 + air +	light,	H_2O,	CO_2,	+	0.46	6.8-	-----	-21.5	-28.3	2.2	
3b 5% CO_2 + glucose[c]	glucose	glucose	glucose	+	0.41	7.3	-32.4±0.4	-21.7	-29.5	4.4	+6.5, -2.9
4a BG11 + air + 5% CO_2	light	-----	CO_2	+	0.04	6.8-	-20.8±0.3				+18.1
4b + DCMU				+	0.04	7.3	------	-10.8	-18.8		
5a BG11 + air + 5% CO_2	light,	glucose	CO_2,	+	0.09	6.8-	-14.8±0.3	-21.0	-29.0	2.0	+24.1, +14.2
5b + DCMU + glucose	glucose		glucose	+	0.10	7.3	-----	-21.0	-29.0	2.0	
6a BG11 + N_2 + DCMU	light,	glucose	glucose	-	0.31	6.8-	-12.7±0.2	-10.6	-18.6		+26.2, +5.9
6b + glucose	glucose			-	0.03	7.3	-----				
7a BG11 + N_2 +	light,	H_2O,	glucose	-	0.30	6.8-	-16.7±0.2	-10.5	-18.5		+22.2, +1.8
7b glucose	glucose	glucose		-	0.20	7.3	-13.8±0.2	- 7.5	-15.5		+25.1, +1.7
[d]8a BG11 + air + 5% CO_2	-----,	glucose	CO_2,	+	0.12	6.8-	-13.5±0.3				+25.4
8b + dark + glucose	glucose		glucose	+	0.13	7.3	-13.6±0.2	-15.6	-23.6	1.2	+25.3, +10.0
9a BG11 + air + CO_2	-----	-----	-----	-	----	5.50-	-----	-17.9	-25.9	2.5	
9b (blank)						5.73	-----	-17.9	-25.9		
Inoculum	-----	-----	-----	-	0.18	----					

*These are amounts of CO_2 (or Carbon) the millimoles per liter representing the total CO_2/HCO_3^-. The glucose is present as 4 g/l = 132 mM Carbon.

[a]See appendix.

[b]delta ^{13}C was $-38.9^{o}/_{oo}$ (parts per thousand).

[c]delta ^{13}C was $-9.0^{o}/_{oo}$,

[d]Run only 2 days.

[e]Lower limit with respect to tank CO_2, upper limit with respect to medium CO_2, as measured

ratios was to be believed. Since both our method of periodically gassing the media and our method for processing the media may have been flawed (see below), it is not possible to report accurate values for carbon isotope fractionation by *Phormidium*. Nevertheless, we believe our qualitative conclusions, which follow, are valid:

Phormidium luridium can use isotopically heavy glucose as a carbon source, as indicated by the resulting isotopically heavy cell mass.

Although it is an aerobic organism, *P. luridium* grows better under microaerophilic conditions.

P. luridium, when confronted with both CO_2 and glucose as available carbon sources, incorporated both the isotopically light CO_2 and the heavier glucose; however, it did not grow faster than under autotrophic conditions.

There was no evidence that *P. luridium* could use glucose as an electron donor in photosynthesis when living aerobically. It was not definitively demonstrated that *P. luridium* can use glucose as an electron donor if grown anaerobically.

Although *P. luridium* was able to use glucose as a carbon source in the presence of O_2, cultures did not increase in cell mass when deprived of photosystem II by introduction of DCMU. O_2 may inhibit glucose fermentation or some aspect of photosystem II may be required for growth under these conditions.

It has been suggested that O_2 may decrease carbon fractionation by inhibiting RuBP carboxylase (Pardue, *et al*, 1975). Since fractionation occured in the presence of O_2, our experiments seem to rule out this as an important effect in *P. luridium*.

SUMMARY

(Wolfgang Krumbein)

Field Work

Isotope fractionation experiments showed that field communities tend to be richer in ^{13}C, that is, isotopically heavy relative to pure cultures of their members. Fractionation values reported in the literature for cyanobacteria and the other microorganisms that we sampled can be very high in ^{12}C. We interpret this as being due to the closed system effect, in which growth outstrips the CO_2 supply, forcing the organisms to make use of CO_2 supplies that are not in equilibrium with the atmosphere. This interpretation is supported by the following observations:

Thiothrix, living in the most open environments in Alum Rock Park, was isotopically the lightest sample studied. *Thiothrix* that were clumped were isotopically heavier than those that trailed in the stream.

The motility-selected cyanobacteria, living in the second

The motility-selected cyanobacteria, living in the second most open environments, were the next isotopically lightest.

The isotopic fractionation was least among organisms of the salt marsh mats. These communities were probably CO_2-limited, as bicarbonate concentrations were far lower than those of the Alum Rock Park site. The simplest interpretation is that these mats were forced to use locally scarce and heavy CO_2.

Field communities tended to be isotopically heavy relative to fossil sedimentary reduced carbon. The reason for this discrepancy and its relation to factors such as environment of deposition and diagenesis is under discussion. Clearly the final isotopic value of carbon in sediment may be related at least to the species in the community, the extent of autotrophic metabolism by those species, the relative population densities, the exchange of CO_2 with the atmosphere and other factors. A direct comparison of the isotope fractionation values of modern microbial mats to those taken from organic matter in fossil stromatolites, cherts, and shales is not yet possible.

Fractionations by purple and green sulfur bacteria were indistinguishable from those by cyanobacteria in the same environment.

Thiothrix fractionated on the order of approximately *delta* $^{13}C = -20\ ^{o}/_{oo}$, which is suggestive of autotrophy. Its carbon source is apparently CO_2 incorporated by the RuBP carboxylase cycle. Both sulfide oxidizing and phototrophic bacteria may be important sources of ^{12}C enrichment seen in the carbon of sedimentary rocks.

The placement of glass wool on microbial mats proved to be a useful technique for concentrating and separating into layers phototrophic members of mat communities. The glass wool technique was especially useful for concentrating pigmented gliding bacteria like *Oscillatoria* sp. that entered it quickly.

Laboratory Work

Our laboratory experiment with *Phormidium luridium* led us to the following conclusions:

P. luridium apparently cannot grow photoorganotrophically in an oxic environment.

P. luridium grows best in an anoxic environment where it may grow photoorganotrophically or photoautotrophically.

P. luridium can ferment glucose in an anoxic environment, but can neither respire nor ferment it in an oxic one.

Free oxygen concentration has, at most, only a minor influence on carbon isotope fractionation in *P. luridium*. The uptake of CO_2 involves the enzyme of RuBP carboxylase in the organism.

The *delta* ^{13}C $^{o}/_{oo}$ value for cell material from *P. luridium* ranged from -12.7 (anaerobic, N_2 + DCMU + glucose) to -55.7 (anaerobic, N_2 + 5 percent CO_2) depending on the growth conditions. The relative fractionation varied from almost none to over 25 percent.

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APPENDIX I
CYANOBACTERIAL MEDIA AND TECHNIQUES

Our composition of Media BG 11, ASN III, BG 11^{-} and ASNIII^{-} is according to Rippka, *et al* (1979).
$^{-}$ indicates omission of $NaNO_3$.

Prepare Stock Solutions:

$NaNO_3$: 150 g/1
$K_2HPO_4.3H_2O$: 4 g/100 ml
$MgSO_4.7H_2O$ 350 g/1
$CaCl_2.2H_2O$: 50 g/1
Fe Solution
Citric acid, ferric ammonium citrate, EDTA (disodium magnesium salt): 600, 600 and 100 mg/100 ml, respectively.
Na_2CO_3: 2 g/100 ml
Trace metal mix A5 + Co:
H_3BO_3 2.86 g/1, $MnCl_2.4H_2O$: 1.81 g/1,
$ZnSO_4.7H_2O$: 222 mg/1, $Na_2MoO_4.2H_2O$:
390 mg/1, $CuSO_4.5H_2O$: 79 mg/1,
$Co(NO_3)_2.6H_2O$: 49 mg/1.

To prepare 1 liter of the medium: put the following amounts of stock solutions into 750 ml H_2O, adjust the pH to 7.0 using HCl and fill it up to 1 liter:

Solution	BG 11	MN	ASN III
$NaNO_3$	10 ml	5 ml	5 ml
$K_2HPO_4 \cdot 3H_2O$	1 ml	0.5 ml	0.5 ml
Na_2CO_3	1 ml	1 ml	1 ml
$MgSO_4 \cdot 7H_2O$	0.2 ml	0.1 ml	10 ml
$CaCl_2 \cdot 2H_2O$	0.7 ml	0.4 ml	10 ml
Fe-solution	1 ml	0.5 ml	0.5 ml
Trace metal mix	1 ml	1 ml	1 ml
NaCl (salt)	-	-	25 g
Sea water	-	750 ml	-
Distilled water	750 ml	-	750 ml

To prepare solid media, put 10 g of purified agar into the medium.

Special Media For Cultures

Test for purity of culture by supplementing the solid medium with 2 g glucose and 0.2 g casamino acids, and looking for heterotrophs.

Test for vitamin B_{12} requirement by adding 1 ml of a solution containing 1 mg/100 ml of B_{12} to the sterilized medium, using a syringe equipped with a sterile filter.

Test for photoheterotrophy by supplementing the medium with 5 g glucose, fructose, sucrose, ribose or 1 g glycerol, acetate or glycolate.

Medium D

	Final concentration
Double distilled water	1,000 ml
NTA (nitrilotriacetic acid)	0.1 g
Micronutrient solution	0.5 ml
$FeCl_3$ solution (0.29 g/liter)	1.0 ml
$CaSO_4 \cdot 2H_2O$	0.06 g
$MgSO_4 \cdot 7H_2O$	0.10 g
NaCl	0.008 g
KNO_3	0.010 g
$NaNO_3$	0.70 g
Na_2HPO_4	0.11 g
Agar	15 g

This medium has a pH of 3. Adjust the pH to 8-8.2 with a 1-2 *M* NaOH solution. Add agar after the pH adjustment. The normal medium before autoclaving has a pH of 8.2. After cooling and complete clearing, our pH was 7.5-7.6.

Micronutrient solution

Double distilled water	1,000 ml
H_2SO_4 (concentrated)	0.5 ml
$MnSO_4 \cdot 7H_2O$	2.28 g
$ZnSO_4 \cdot 7H_2O$	0.50 g
H_3BO_3	0.50 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g
$CaCl_2.6H_2O$	0.045 g

Reference

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria, J. General Microbiology, 111:1-61.

CHAPTER II
ANOXYGENIC PHOTOTROPHIC BACTERIA

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INTRODUCTION TO GREEN AND PURPLE PHOTOSYNTHETIC BACTERIA

The initial aim of this group was to introduce young scientists of different fields to the techniques of recognition, enrichment, isolation, identification and maintenance of anoxygenic photosynthetic bacteria (Stanier *et al.*, 1981). Pure cultures of *Rhodopseudomonas*, *Rhodospirillum* (Rhodospirillaceae), *Chromatium*, *Thiocapsa*, *Ectothiorhodospira* (Chromatiaceae), and *Chlorobium*, *Prosthecochloris* (Chlorobiaceae) were obtained from field samples, including samples from Big Soda Lake (Nevada) and Mono Lake (California), both extremely alkaline hypersaline environments.

Although purple and green bacteria are of central importance to phylogenetic studies, their predominantly anaerobic phototrophic way of life renders them difficult to isolate and maintain in pure culture. This holds true especially for the phototrophic sulfur bacteria (Chromatiaceae, Chlorobiaceae) which consume reduced sulfur compounds (*e.g.* sulfides) at low levels but cannot tolerate them in high concentrations.

To become familiar with the techniques involving anoxygenic phototrophic bacteria, pure cultures from the collection of the Department of Microbiology, University of Bonn, Germany, were used, in addition to the enrichments and isolations obtained at the field sites during the course.

Samples containing red, purple, pink, or olive-green material were collected at three sites in Alum Rock Park, and at four sites at the salt marsh. For the isolation of *Ectothiorhodospira* strains, alkaline and highly saline samples were obtained from Australia and western North America as noted.

Based on the isolations and the mastery of techniques the following studies were undertaken:

We measured the *delta* ^{13}C of growing *Chlorobium vibrioforme*, which has a complex "reverse Krebs cycle" mechanism of CO_2 assimilation. This organism proceeded through a typical batch culture growth curve from rapid to slow growth. Preliminary results indicated that the bacteria fractionate carbon less than -5 $^{o}/_{oo}$, a result which may be related to their ancient origin.

We studied the fractionation of nitrogen and carbon with growing cultures of Rhodospirillaceae under photoheterotrophic conditions (light, N_2) relative to chemoheterotrophic conditions.

We studied the influence of salinity on growth and the sulfide turnover in Chlorobiaceae and Chromatiaceae from fresh water (Alum Rock Park) and from the salt marsh (Embarcadero Road).

We compared the absorption spectra of photosynthetic bacterial cultures after different preparation methods to determine the most reliable methods to identify the different genera and species.

ENRICHMENT, ISOLATION AND GROWTH OF PHOTOTROPHIC BACTERIA

Rhodospirillaceae: *Rhodopseudomonas*, *Rhodospirillum*

(Dorothy Read)

Samples were collected from the two field sites and enrichments for non-sulfur purple phototrophic bacteria (Rhodospirillaceae) were carried out in three media, which all contained sulfate and an organic carbon source, but differed in pH (5.5, 6.8, or 7.3) and carbon source (acetate or succinate) (see Appendix II for composition and preparation of media). Each enrichment culture consisted of a 125 ml bottle inoculated with a few drops of the field sample and filled with medium, leaving only a small air bubble to accomodate pressure changes (Biebl and Pfennig, 1981). The bottles were incubated facing a 100-Watt incandescent bulb at a distance of 35-45 cm, resulting in an estimated illumination of 200 lux and a measured temperature of 28-32° across the bottles. Growth, as evidenced by visible turbidity and pinkish or brownish color, was observed in three to five days in all the enrichments in the pH 7.3 acetate medium, and in five to seven days in the enrichments in pH 6.8 succinate medium (see Table II-I). However, no growth was observed in the pH 5.5 medium even after 30 days, suggesting that acidophilic Rhodospirillaceae were not present in significant numbers at the field sites sampled. This is consistent with the neutral to

somewhat alkaline range of the pH measurements of these sites, noted above.

Table II-I Results of Enrichment Cultures

Field Sample	Enrichment Medium	
	Succinate pH 6.8	Acetate pH 7.3
F1	red-brown	yellow-brown
F2	green	greenish-brown
F3	yellow-brown	brownish yellow-green
M1	pinkish-brown	pink
M2	brownish-green	red-brown
M3	pinkish brown	red brown

F1,2,3 = freshwater samples from Alum Rock Park. See Fig. I-I.
M1,2,3 = marine samples, salt marsh, see Fig. I-I for sites.
All samples showed growth (obvious turbidity) within a week or so.

Pure cultures were obtained by plating on the same medium and picking individual colonies, either by quadrant streaking or by suspension in distilled water, dilution, and spreading. Plates were incubated in anaerobic jars under the same illumination as the liquid culture. Purified isolates were identified by the criteria of colony appearance, microscopic morphology (cell size, shape, motility, aggregates, and inclusions), and, when necessary to make a species determination, by carbon source utilization or absorption spectrum of sucrose-lysed cells (see Appendix II for media and methods). In most of the enrichments, one colony type predominated and was purified.

The pure cultures obtained are shown in Table II-II with their identification. Attempts to grow these organisms on molecular nitrogen as the only nitrogen source were unsuccessful within the available time (maximum two weeks).

Table II-II Pure Cultures of Rhodospirillaceae

Enrichment Culture	Characteristics and Identification
F1 on Ac 7	Colonies: round, smooth, red-brown. Microscopic: short, almost coccoid rods, about 1 x 2μ; highly motile; some groups of two or four; a few zigzag chains of four to ten. Negative stain with India ink showed transparent capsule. Carbon source utilization: growth on 0.1% acetate, no growth on 0.2% ethanol. Identification: Rhodopseudomonas capsulata
F2 on Ac 7	Colonies: round, smooth, rust-brown Microscopic: small motile rods, slightly pear-shaped, appearance of budding. Tentative indentification: R. blastica (described recently by Eckersley and Dow 1980)
F3 on Ac 7	Colonies: smooth, round, dark yellow-brown Microscopic: short motile rods about 0.5 x 1.5μ, some appearance of budding. Identification: R. palustris
M2 on Ac 7	Colonies: round, smooth, red-orange. Microscopic: short motile rods 1.5 - 2μ long, some movement in circles, many pairs appear budding. Dark inclusions one or two per cell near end(s) of cells. Identification: Rhodopseudomonas palustris
F1 on Succ	same as F1 on Ac 7. (R. capsulata)
M3 on Succ	Colonies: round, smooth, dark yellow-brown. Microscopic: very large spiral cells, motile, about 1μ x 15 - 20μ, up to 30μ long. Absorption spectrum of sucrose-lysed cells: maxima at 585 nm and 460, 490, 525 nm (triple peaks). None at 550 nm. Identification: Rhodospirillum molischianum

Cells taken from plates incubated in anaerobic jars. Microscopy of cells in wet mount in distilled water observed by phase contrast with ocular micrometer at 1000X magnification.

Ac 7 = acetate pH 7.3
Succ = succinate pH 6.8

Ectothiorhodospira Species

(Jaap Boon)

Field samples from hypersaline ponds at West Dunbarton Bridge, from the alkaline lakes Mono Lake, California, and Big Soda Lake, Nevada, from the cyanobacterial mats of Laguna Figueroa, Mexico and Shark Bay, Australia, and from the Great Salt Lake, Utah, were incubated in media suitable for growth and isolation of *Ectothiorhodospira* species. Several strains of *Ectothiorhodospira* were isolated from the alkaline lakes, Mono and Big Soda Lakes.

A pure culture of *Ectothiorhodospira halophila* was maintained on the medium of Imhoff and Trueper (1977). Attempts were made to grow these bacteria on the same medium without organic substances (yeast extract and sodium succinate) with

the aim of preparing them for *delta* ^{13}C analysis by autotrophic growth on CO_2 as the sole carbon source, but very poor growth was obtained on these media.

Media used for growth and isolation of *Ectothiorhodospira*

The medium in which *Ectothiorhodospira halochloris* was isolated by Imhoff and Trueper (1977; 20 percent NaCl) was used for maintenance of the *Ectothiorhodospira halophila* culture.

For greater versatility in medium preparation, the mineral part and the organic part of the medium were prepared separately and combined when a medium plus organic components (yeast extract, vitamins and sodium succinate) was needed for enrichment or isolation. The medium used for enrichment at 6.6 percent NaCl was derived from that of Imhoff and Trueper (1977) by using one third of the amounts of NaCl and Na_2SO_4.

The choice of 6.6 percent salinity was based on determinations of major ions in the sulfide rich bottom water of the Big Soda Lake (see Table II-III). The pH of the medium was 8.7. A 3 percent salinity medium described for isolation of phototrophic sulfur bacteria by Trueper (1970) was used in enrichments from Mono Lake. This medium was enriched with yeast extract, vitamins and sodium succinate in concentrations similar to those in the Imhoff and Trueper (1977) medium.

The pH was adjusted to 10, since the measured pH of the Mono Lake water sample was pH 10.5. At this pH, a precipitate developed, but it did not affect the growth of the bacteria.

For agar shake dilution experiments, the methods given by Trueper (1970) were followed. It is advisable, however, to increase the amount of agar at high salinity and high pH to a final concentration of 1.2 percent. All cultures were incubated at room temperature and illuminated by ordinary white light bulbs.

Table II-III Major Ions (mg/l) Throughout Water Column of Big Soda Lake (pH 9.7)

Component	Surface Water	Bottom Water
Na^+	8050.0	27000.0
K^+	315.0	1130.0
Cl	7000.0	28000.0
Br^-	16.0	59.0
T^-	1.7	4.0
Mg^{++}	148.0	5.6
Ca^{++}	5.0	0.8
Sr^{++}	1.4	0.5
SO_4^-	5600.0	6500.0
B^-	42.0	190.0
Alkalinity (as HCO_3^-)	4000.0	24000.0
Fe^{+++}	0.1	0.1
NH_4^+	0.1[3]	45.0
Diss. Org. Carbon[2]	20.0[3]	60.0[3]
S^-	0.0	200.0
Other reduced sulfur	0.0	200.0
SiO_2	4.0[3]	210.0
Salinity (estimated)	21.13	62.69

[1]Data courtesy of Dr. R. Oremland, USGC.

[2]Dissolved organic carbon.

[3]Seasonally affected.

Samples available:

Big Soda Lake samples were provided by R. Oremland (USGS). Samples available were: a Winogradsky column enrichment culture with lake water and mud, and pure lake water collected in February 1982 (coded OR). The Mono Lake water was collected in July 1982 by H. G. Trueper.

The Great Salt Lake sample contained mainly mud and some supernatant with pink microorganisms. The Shark Bay sample was a carbonate sand containing green coccoid cyanobacteria and purple bacteria. The sample was dry and had been exposed to the atmosphere.

The Laguna Figueroa sample was an enrichment kept in the light for two years in the Biology Department of Santa Clara University by G. Tomlinson. The sample contained reddish purple

bacteria which were thought to be living *Ectothiorhodospira spp.* Water near the NaCl saturation point was collected in an orange colored pond at West Dunbarton Bridge, San Francisco Bay. Other samples from this location were taken from old salt ponds including a gypsum crust containing cyanobacteria underlain by a purple layer, and mud.

Results from enrichments for *Ectothiorhodospira*

No growth was obtained in samples from Great Salt Lake, Shark Bay, Australia, Laguna Figueroa and the West Dunbarton Bridge. *Ectothiorhodospira spp.* were enriched in samples from Big Soda Lake and from Mono Lake. The Big Soda Lake samples grew to high density in a few days on the 6.6 percent NaCl medium supplied with the organic compounds at a pH of 8.7. Some growth was obtained in the inorganic medium. Microscopy of cultures from these samples showed rod-shaped, very motile cells (0.9 μm wide), immotile ellipsoidal cells with gas vacuoles (long axis 2 μm) and spirochetes (18 μm long). After 4 weeks of incubation, some growth was obtained in 20 percent salt supplied with organic components. *Ectothiorhodospira spp.* were cultured through agar shake dilutions on organic-free and organic-containing media in 6.6 percent NaCl enrichments. Similarly, the Big Soda Lake water sample containing visible amounts of *Ectothiorhodospira vacuolata* (ovoid cells with vacuoles) was cultured through agar shake dilution on mineral medium at 6.6 percent NaCl.

Ectothiorhodospira halophila, a pure halophilic strain, was successfully maintained on the medium of Imhoff and Trueper (1977). This medium contains 20 percent NaCl, yeast extract, sodium succinate and vitamins. Very poor growth was obtained on inorganic media. After several transfers on this medium, however, pinkish suspensions were visible in the culture flask which indicated that some growth was possible under these conditions. No attempts were undertaken to grow sufficient cells for *delta* ^{13}C analysis on this high salt medium.

Two single colonies, growing in the first dilution of the Big Soda Lake water sample, were selected and used for isolation. They were grown in 6.6 percent NaCl supplied with organics. One of these colonies (Coded OR-A) consisted of nonmotile gas vacuolated ellipsoidal cells which formed a fine pink suspension. These organisms were tentatively identified as *Ectothiorhodospira vacuolata*. The others (Coded OR-B) were motile rods, which formed red clots of cells. These organisms were tentatively identified as *Ectothiorhodospira mobilis*.

Two colonies were isolated from the 7th dilution of the enrichment on 6.6 percent NaCl without organics. They were transferred to 6.6 percent NaCl medium with organics. The one coded S6-A was a pink suspension in which many *E. vacuolata* and some *E. mobilis* were seen. The one coded S6-B showed red clots, which prefer the glass surface for growth. The isolate was a pure culture of motile rods.

The colony isolated from Big Soda Lake in the 7th dilution growing on 6.6 percent NaCl plus organics, was transferred to the same medium. This isolate contained both motile rods and gas vacuolated cells.

Lake water from Mono Lake that contained no visible microorganisms visible) was incubated in the 6.6 percent NaCl medium plus organics, and in 3 percent NaCl medium plus organics. Both media were adjusted to pH 10 with 2*M* Na_2CO_3, because the pH of the lake water was 10.5.

The enrichment on 3 percent NaCl was very successful. Within two days, a massive development of red colored cells was observed which formed clots sinking to the bottom of the flask. A similar, but slower growth pattern was observed in the 6.6 percent NaCl medium plus organics. No growth was obtained on 20 percent NaCl media. The only microorganisms in both enrichments were motile rods.

The results of these enrichments and isolations support the hypothesis of Trueper and Imhoff (1981) that in alkaline, strongly saline environments *Ectothiorhodospira* species are the prevailing purple sulfur bacteria. *Ectothiorhodospira* species so far have only been isolated from rather shallow soda lakes and salt flats. They were also enriched over a six month period in a light-incubated sample bottle collected by E.S. Barghoorn and C.C. Lenk at Carbla Point, Shark Bay, W. Australia. They were identified during the summer course by H. Trueper.

Chromatiaceae and Chlorobiaceae: *Chromatium*, *Thiocystis*, *Thiocapsa*, *Prosthecochloris*, *Chlorobium*

(Deborah B. Craven)

Samples from Alum Rock Park and the salt marsh were inspected by light microscopy. They all contained several morphologically distinguishable species of Chromatiaceae and Chlorobiaceae (Pfennig and Trueper, 1981). The pink to brownish

material from the freshwater sites at Alum Rock Park was dominated by *Chromatium vinosum* and *Chlorobium* species. The purplish-pink samples from the salt marsh contained large and small cell *Chromatium* species, *Thiospirillum*, *Thiocapsa* and *Thiocystis*, as well as unidentified Chlorobiaceae.

Liquid enrichment cultures with Pfennig's medium + 3 percent NaCl (see Appendix II) were obtained from all samples. The relatively low light intensity favored Chlorobiaceae over Chromatiaceae. Therefore, direct inoculations of fresh sample materials into agar shake dilution series (Pfennig's medium + 3 percent NaCl) were done in addition, as these preserve the original ratio of purple to green sulfur bacteria in the field sample.

From these direct inoculations and the liquid enrichments we obtained, by repeated agar shake dilution series, pure cultures of the following species:

From Alum Rock Park:

Chlorobium limicola
Chromatium vinosum

From the salt marsh:

Chromatium buderi
Chromatium vinosum
Thiocystis violacea
Thiocapsa roseopericina
Rhodopseudomonas sulfidophila
Rhodospirillum sp.
Prosthecochloris aestuarii

The identification of these species was carried out according to Trueper and Pfennig (1981). The species isolated from the salt marsh generally agreed with those found by Trueper (1970) in different marine environments. Typical for these environments are *Chromatium buderi*, *Prosthecochloris aestuarii* and *Rhodopseudomonas sulfidophila* as NaCl (3 percent) requiring bacteria. *Chromatium vinosum*, *Thiocystis violacea*, and *Thiocapsa roseopericina* are also very common in marine environments.

Chromatiaceae and Chlorobiaceae

(Alejandro López Cortés)

Samples were taken from the field site and observations of pink to purple red masses with different concentrations of sulfur compounds were made. Cultures were prepared in Pfennig's medium (Trueper, 1970). The water and mud samples were inoculated into screw capped bottles containing Pfennig's medium, and incubated in light (40-50 foot candles) at room temperature. The cultures were then prepared by agar shake dilution (Trueper, 1970; see Appendix II) and single colonies were removed. The colony was suspended in 1 ml of sterile medium, and the whole agar dilution series was repeated (see Appendix II).

The isolated colonies were identified by morphological characteristics and physiological properties including their *in vivo* absorption spectra.

At the Alum Rock Park sites the temperature was 28° and the pH was 6.8. *Thiothrix* filaments associated with cyanobacterial black nodules were observed. The partial isolation of anoxygenic photosynthetic bacteria was observed to include *Chromatium*, *Thiocystis*, and *Chlorobium*. The motile *Chromatium* was purple-red, rod shaped and 2.0-2.7 μm wide by 3.0-4.5 μm long. The motile *Thiocystis* (2.0-3.1 μm) was pink-red and spherical. The *Chlorobium* was yellow-brown, rod shaped and 1.0 μm wide by 1.5 μm long.

The temperature of the salt marsh was found to be 32°-34° C. Partial isolation of the samples from this site consisted of *Chromatium buderi* and *Chlorobium sp.* The *Chlorobium* cells, 0.8 μm by 1.5 μm, are green in suspension. The *Chromatium buderi* were motile rods 4.4-4.8 μm wide by 6.7-9.6 μm long (Fig. II-1b). They were the color of the suspension (purple-violet) and bchl *a* was present (Table II-IV and Fig. II-2). A Carey 14 spectrophotomer was used to obtain the absorption spectrum.

Fig. II-1 Mixed Culture with *Chlorobium sp.* (A) *Chromatium buderi* (B)

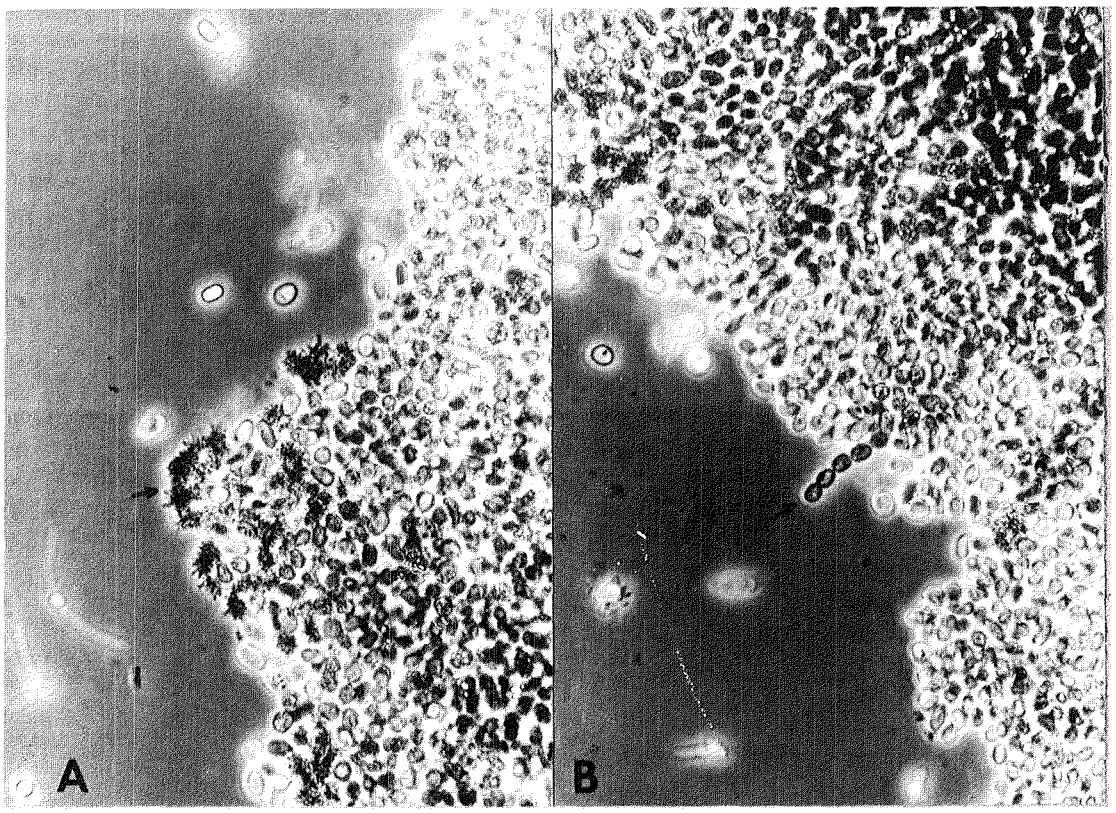


Figure II-2

Absorption Spectrum of a Mixed Culture of *Chromatium buderi* and a *Chromatium* sp.

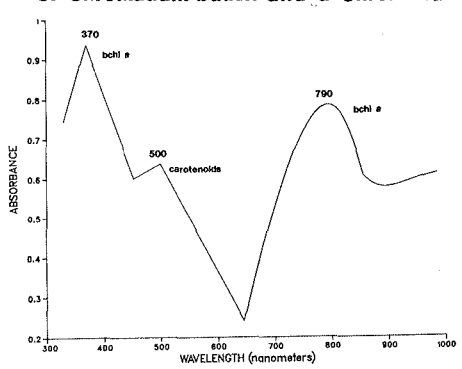


Table II-IV Mixed Culture of a Cell Suspension of a *Chromatium buderi* and a *Chlorobium* sp.

Wavelength (nm)	Absorbance %
325	0.72
350	0.82
270	0.93 bchl a
400	0.76
450	0.58
500	0.63 carotenoids
530	0.57
550	0.48
570	0.46
610	0.33
650	0.22
730	0.59
750	0.73
790	0.78 bchl a
800	0.76
850	0.59
900	0.58
925	0.60
950	0.61
975	0.63

CARBON FRACTIONATION DURING CARBON DIOXIDE FIXATION

(Jeffrey P. Chanton, Jaap Boon, Karinlee Kneller)

Introduction

Recent investigations on the biochemical mechanisms for carbon dioxide fixation in the Chlorobiaceae have shown that in these organisms the fixation of CO_2 does not occur by ribulose bisphosphate carboxylase (RuBP) and the Calvin cycle. Moreover, Fuchs, *et al.* (1980 a,b) and Ivanovski, *et al.* (1980) demonstrated that the principal mechanism of CO_2 fixation in this group is via the reverse tricarboxylic acid cycle as orginally proposed by Evans *et al.* (1966). This pathway contains four distinct CO_2 fixation steps, mediated by the following enzymes: pyruvate synthase, alpha ketoglutarate synthase, and isocitrate dehydrogenase, as well as C_3 and C_1 carboxylation. These findings are remarkable, because the purple phototrophic sulfur bacteria Rhodospirillaceae and Chromatiaceae (including *Ectothiorhodospira*) principally fix CO_2 via ribulose bisphosphate carboxylase, *i.e.* the Calvin cycle.

There are few studies investigating the nature of the isotopic fractionation of carbon by the purple and green sulfur bacteria (Quandt, *et al.*, 1977, Sirevag, *et al.*, 1977, Wong, *et al.*, 1975, and Abelson *et al.*, 1961). In addition, these studies predated the recent evidence for the presence of an alternative CO_2 fixation pathway, *i.e.* the reverse TCA cycle, in the Chlorobiaceae. Consequently, a detailed study of the carbon isotope fractionation in this group of phototrophic bacteria, paying careful attention to their phase of growth in batch culture, to closed system isotope effects, and to the fractionation between the bicarbonate and dissolved CO_2, seemed necessary. We also measured the carbon fractionation of several purple sulfur bacteria for comparison, and of an enrichment culture of the green sulfur bacterium *Prosthecochloris aestuarii* which was isolated from the salt marsh.

Methods

Four pure cultures of phototrophic sulfur bacteria, *Chromatium warmingii*, *Chromatium vinosum*, *Ectothiorhodospira shaposhnikovii* and *Chlorobium vibrioforme* were grown in 125 ml batch cultures according to the methods of Trueper (1970). To reduce any potential interfering isotopic fractionation of

carbon, it was necessary to exclude all extraneous sources of organic carbon from the growth media. Consequently, the following precautions were taken:

> Hydrochloric acid was substituted for EDTA to maintain the solubility of the trace metals (Pfennig and Trueper, 1981), Teflon spacers were used to line the culture bottle caps, and Teflon tubing was used in place of rubber tubing on the media dispensing apparatus.

Since vitamin B_{12} is a necessary growth requirement for these bacteria, it was not possible to omit it from the medium. However, it is believed that vitamin B_{12} at 0.02 mg per liter concentration would not substantially interfere with the isotopic signature of the medium nor the cells during growth. Each culture of *Chlorobium vibrioforme* was supplemented with 1 ml of 10 percent thiosulfate solution ($Na_2S_2O_3.5H_2O$) to provide an additional electron source. In addition to the four pure cultures above, an enrichment culture of *Prosthecochloris aestuarii* (isolated from the salt marsh) was grown in the described medium without the thiosulfate supplement. All cultures were incubated at ambient room temperature and under an incandescent light source. The green sulfur bacteria were grown at about 30 lux and the purple sulfur bacteria were grown at 60 lux.

Chlorobium vibrioforme cultures for the short term growth study were harvested after 3, 13, 24, and 48 hours of incubation. Parallel *C. vibrioforme* cultures for the long term growth studies were harvested after 1, 3, 5, 8, and 10 days of incubation. At the time of harvesting, a 5 ml aliquot was removed with a syringe for the medium CO_2 analysis (see below), and another 10 ml aliquot was removed for pH and for optical density determinations (at 620 nm) The remainder of the 125 ml culture was reserved for the cell CO_2 analysis.

Initially, the 5 ml medium samples were filter sterilized through a 0.22 μm cellulose acetate filter (Millipore GS) that was pre-washed with 0.1 N HCl (2 ml) and filter rinsed with distilled water (10 ml). The filter sterilized medium was then injected through a butyl rubber septum into a N_2 flushed serum vial and stored frozen until the total CO_2 analysis could be performed. For the total CO_2 analysis, 0.5 ml aliquots of the filtered medium were injected into an evacuated round bottom reaction flask containing 2 ml of 2*M* $CuSO_4$ and 2 ml of 2*M* H_2SO_4. The $CuSO_4$ was added to precipitate the sulfide ion, as H_2S gas may interfere with mass spectrophotometric determinations. Because it was believed that some of the medium CO_2 was being lost to the gas phase in the serum vial, subsequent samples were made alkaline with 1N

NaOH (1 ml) just prior to the transfer to the round bottom flask. Because of the relatively large surface area to volume ratio of the reaction flask, some of this medium was retained by the inner and upper walls of the reaction vessel. During the evacuation procedures for the total CO_2 gas analysis, this residual medium released CO_2 in a non-quantitive manner, reducing the reproducibility of the total CO_2 determinations.

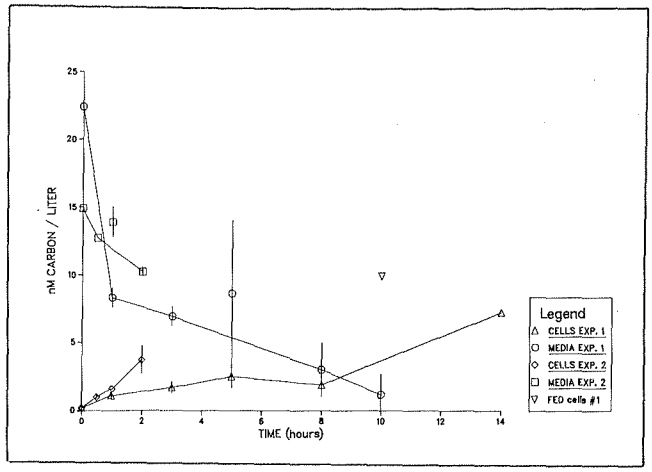
Further modifications of the medium sampling procedure and total CO_2 analysis were then employed. One ml aliquots of medium were transferred directly to the sample serum vial described above. Furthermore, the gas analysis line was modified by removing the reaction flask and replacing it with a hypodermic syringe needle. This hypodermic needle was pushed through the septum of the vial containing the medium just prior to the gas analysis. These modifications reduced the number of transfers to one, and reduced the probability of inadvertent degassing and increased the reproducibility of the total CO_2 determinations.

The cultures of the phototrophic bacteria were collected by centrifugation (Sorvall RC-II) at 11,000 rpm for 10 minutes. They were resuspended in an isotonic washing solution, and then recentrifuged. The pellets were stored frozen, then freeze-dried and weighed for dry weight determinations. The medium and cell samples for the isotopic analysis were processed according to the methods of DesMarais (see Appendix IV).

Results and Discussion

Figure II-3 shows the growth curves for the two *Chlorobium vibrioforme* growth experiments. Experiment 1 was run over a period of 14 days while the ambient temperature was about 25° C, whereas Experiment 2 was shorter (48 hours) and the ambient temperature was 28° C. Both optical density and the concentration of organic carbon in *mM* per liter are shown on the graph. The concentration of organic carbon was calculated from the culture dry weight and its percentage carbon. The culture marked "fed" on the graph received an additional ml of sodium thiosulfate solution two days before it was harvested. The growth curve of unfed cultures in Experiment 1 shows a maximum on day 5, then a decline, and a second maximum on day 14. Prior to day 14, elemental sulfur was observed in all cultures when the cells were collected; however, on day 14, it was noticed that no elemental sulfur was present. It would appear from these observations that the bacteria had a lag period while switching from sulfide and thiosulfate as electron donors to elemental sulfur. It is also apparent from Figure II-3 that the growth rate in the second experiment was higher than the growth rate in the first experiment.

Figure II-3 Growth of *Chlorobium vibrioforme* and Decreases in Media Inorganic Carbon in Growth Experiments One and Two



Total dissolved inorganic carbon measurements and isotopic measurements of the dissolved inorganic carbon and cell carbon are shown in Tables II-V and II-VI. The *delta* ^{13}C of

Table II-V Carbon Isotope Fractionation by *Chlorobium vibrioforme* (2 days)

SAMPLE time, hours	pH	%T	mM Cells	mM ΣCO_2	delta $^{13}\Sigma CO_2$ (°/oo) *	delta $^{13}CO_2$ (°/oo)	delta ^{13}C cells (°/oo)
initial medium	6.5	--	---	15.9	-34.6	-38.8	
				13.8	-32.2	-36.4	
				12.9	-32.6	-36.8	
				17.9	-31.7	-35.9	
				16.0	-31.6	-35.9	
$\bar{x} \pm \sigma$				14.9 ±1.9		-36.8 ±1.1	
3 hours	6.7		0.1				-41.6
	6.7	59%	0.3				-41.4
$\bar{x} \pm \sigma$			0.2±0.1				-41.5 ±0.1
13 hours	6.8	35%	0.98	12.71	-30.3	-35.72	-43.5
	7.0	36%	1.06				-45.4
$\bar{x} \pm \sigma$			1.02 ±0.1				-44.4 ±1.3
24 hours	6.8	--	---	14.7	-24.9	-29.0	-46.0
	6.9	--	1.61	13.1	-28.1	-33.9	-46.1
$\bar{x} \pm \sigma$				13.9 ±1.1		-31.4 ±3.5	-46.0 ±0.1
48 hours	6.3	23%	4.4	9.9	-26.9	-30.2	-44.5
	6.4	23%	3.0	10.3	-18.3	-22.1	-45.6
$\bar{x} \pm \sigma$			3.7 ±1.0	10.2 ±0.3		-26.2 ±5.7	-45.1 ±0.1

* °/oo = parts per thousand, temperature = 28°C. Experiment 2

Table II-VI Carbon Isotope Fractionation by *Chlorobium vibrioforme* (14 days)

SAMPLE	pH	%T	mM Cells	mM ΣCO_2	delta $^{13}\Sigma CO_2$ (°/oo) *	delta $^{13}CO_2$ (°/oo)	delta ^{13}C cells (°/oo)
initial a	6.9	--	0.106	22.5	-31.2	-39.2	- 6.5
b	6.9	--	0.301	22.4	-31.3	-39.2	- 6.8
$\bar{x} \pm \sigma$			0.203 ±0.14	22.4 ±0.04		-39.2 ±0.0	- 6.6 ±0.2
day 1	7.2	37%	1.27	7.8	-27.4	-35.3	-35.1
	7.2	36%	1.01	8.8	-27.2	-35.2	-32.2
$\bar{x} \pm \sigma$			1.14 ±0.18	8.3 ±0.7		-35.2 ±0.1	-33.6 ±2.0
day 3	7.1	22%	1.43	7.4	-26.4	-33.0	-39.9
	7.1	18%	2.00	6.4	-25.6	-32.2	-39.8
$\bar{x} \pm \sigma$			1.72 ±0.40	6.9 ±0.7		-32.6 ±0.6	-39.8 ±0.1
day 5	6.8	18%	3.13	4.7	-22.4	-28.1	-39.9
	6.8	18%	1.95	12.4			-39.8
$\bar{x} \pm \sigma$			2.54 ±0.83	8.6 ±5.4			-39.8 ±0.1
day 8	6.7	20%	1.88	1.66	-20.9	-26.2	-39.8
	6.7	17.5%		4.38	-20.3	-25.6	-39.0
$\bar{x} \pm \sigma$				3.02 ±1.92		-25.9 ±0.4	-39.4 ±0.2
day 10	6.1	21%	---	0.157	---	---	-38.8 ±0.2
fed	4.5	17%	9.91	2.27	- 7.5	- 8.39	-37.0 ±0.2
day 14	6.5	17%	7.15	---	---	---	-37.8 ±0.2

* °/oo = parts per thousand, temperature = 25°C. Experiment 1

the dissolved CO_2 was calculated from the pH and the *delta* ^{13}C of the bulk media, which was comprised of dissolved CO_2 and bicarbonate ions. There was an equilibrium isotope effect and the CO_2, which the bacteria assimilated, is about 8 °/oo heavier than the bicarbonate. The tables of Friedman (1977) were used to determine the precise distribution of isotopes in the inorganic carbon phase. The resultant *delta* ^{13}C of CO_2 is plotted in Figures II-4 and II-5, and the concentration of total dissolved inorganic carbon is plotted in Figure II-3. Standard deviations were calculated from replicate cultures. In Experiment 1, there was a large change in the total concentration of dissolved inorganic carbon between the initial time of preparation of the medium and day 1 (Fig. II-3), possibly as a result of outgassing of the medium during inoculation. Through time, the concentration of organic matter in the cells never got higher than one half of the total dissolved carbon concentration in the inital uninoculated medium. It is also likely that some of the dissolved CO_2 was fixed and then excreted by the cells into the medium (Lippert, 1968). However, we made no

attempt to measure dissolved organic carbon.

Fig II-4

Changes in the Isotopic Signature of CO_2 and Cells During Growth of *Chlorobium vibrioforme*.

Experiment One, July 2–16,1982

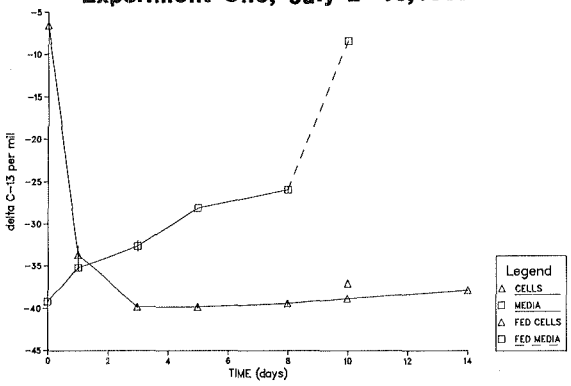
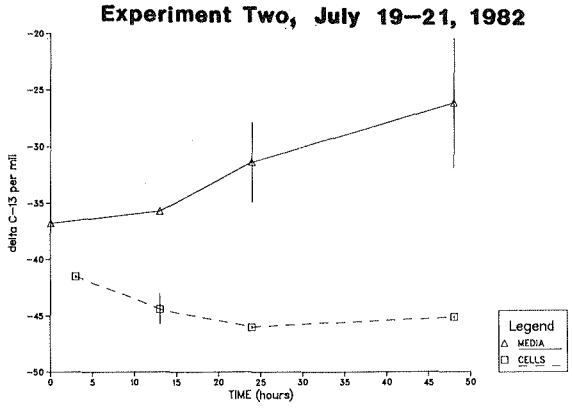


Fig II-5

Changes in the Isotopic Signature of CO_2 and Cells During Growth of *Chlorobium vibrioforme*.

Experiment Two, July 19–21, 1982



In Experiment 2 the rapid decrease in total inorganic carbon was not observed. At the end of 48 hours, 93 percent of the initial medium-dissolved inorganic carbon could be accounted for as a component of total CO_2 and of cells.

The trend of the *delta* ^{13}C was similar in both experiments: the *delta* ^{13}C of the dissolved inorganic carbon increased while the ^{13}C carbon content of the cells decreased, stabilized, and then increased slightly. Fractionation factors were calculated from equations developed for single step reactions in closed systems (Melanders and Sanders, 1980). There are two ways to arrive at the fractionation factor: from the changes in concentration and isotopic composition of the medium in a given time interval, or from the concentration and isotopic signature of the initial medium and of the cells which grew in it. Table II-VII contrasts these fractionation factors calculated by simply looking at the difference of signatures between the CO_2 and the cells. Day 1 was used as the initial sample for long term calculations because of uncertainty about the total CO_2 concentration between day 0 and day 1. The mass and isotopic composition of the cultures at day 1 were removed and fractionation factors were calculated relative to the growing cells.

In Experiment 1, the fractionation factor was very small from day 0 to day 1, but later increased to values of around -10 $^{o}/_{oo}$, and finally reached -25 $^{o}/_{oo}$ when the experiment was carried out to day 14. Basically, Experiment 2 followed the

same trend; the carbon isotope fractionation increased with the age of the culture. Two possible explanations for this phenomenon are:

That the cells released increasing amounts of ^{13}C enriched extracellular products with age and increasing density of the culture, or

That as the growth rate slowed, the CO_2 uptake rate slowed and the cells discriminated more against ^{13}C.

If the first explanation is correct, and the heavy extracellular products were converted to CO_2 and removed from the system, then this mechanism may partially explain why the *delta* ^{13}C content of sediments often decreased with depth, as discussed by Hayes (1983). If the second explanation is correct, perhaps it can be attributed to the variety of enzymes that were available to the bacteria for CO_2 assimilation. The enzymes could fractionate differently, and their relative importance shift during growth of the culture, especially in batch culture.

Table II-VII

Calculated Fractionation Factors for Phototrophic Bacteria

	Closed System Media to Cells ‰*	Closed System Media to Media ‰	By Difference From Graph ‰
Day (Experiment 1)	Chlorobium vibrioforme	T = 25°	
0- 1	+ 1.3	- 4.2	+ 2.5
1- 3	-10.6	-14.5	- 5.0
3- 5	-13.5	-12.3	-12.0
5- 8		- 4.9	-12.75
8-10		+19.2	
5-14	-25.3		
1- 5	-11.1		
1- 8	-14.3		
1-14	-16.5		
Hour (Experiment 2)	Chlorobium vibrioforme	T = 28°	
3-13	- 9.3	-12.4	- 9.0
12-24	-14.2	-70.0	-15.0
24-48	-20.8	-29.5	-19.0
Chromatium warmingii	-20.7	-10.7	
Chromatium vinosum	-16.4	-36.1	
Ectothiorhodospira shaposhnikovii	- 6.9	-16.3	
Prosthecochloris aestuarii	-11.5		

*°/oo = parts per thousand

Fractionation factors for four other species are shown in Table II-VII and II-VIII. Approximately one half of the total CO_2 in the media was used up in each case, except for *Prosthecochloris aestuarii* which used up one quarter of the available CO_2.

Table II-VIII Species Comparison Data

	pH	%T	mM ΣCO_2	delta $^{13}\Sigma CO_2$ (‰) *	delta $^{13}CO_2$ (‰)	delta ^{13}C cells (‰)
C. warmingii	6.7	69%	12.66	-26.9	-32.0	-52.94
pure (27°)	6.6	69%	10.10	-27.4	-32.5	±0.3
			11.38		-32.2	
			±1.8		±0.4	
C. vinosum	7.0	59%	-----	-----	-----	-----
pure (27°)	7.2	54%	10.3	-25.7	-32.3	-52.87
					±0.3	±0.2
E. shaposhnikovii	7.2		10.1	-25.1	-31.7	-43.5
pure (27°)			±0.6	±1.3	±1.3	±0.4
P. aestuarii	6.8	---	15.9	-29.6	-35.1	-48.4
enriched (28°)			±0.3	±0.3	±0.3	±0.1

* °/oo = parts per thousand.

The range of results presented in Table II-VI, II-VII and II-VIII is within the range of results cited in the literature, though the results based upon media changes alone showed the strongest anomalies. Sirevag *et al.* (1977) reported fractionation factors of -12.2 °/oo for *Chlorobium*, and -22.5 °/oo for *Chromatium vinosum* grown in batch culture. Quandt *et al.* (1977) reported fractionations ranging from -2.5 °/oo to -5.2 °/oo for several species of *Chlorobium* including *C. vibrioforme*. They also reported a fractionation of -19.57 °/oo for *Chromatium vinosum*. Wong *et al.* (1975) report a range of fractionation of -15 °/oo to -19 °/oo for *Chromatium vinosum*.

The results for the *Ectothiorhodospira* cell-media calculations were somewhat anomalous, as this bacterium is known to use the Calvin cycle, and thus should fractionate similarly to the *Chromatium* species.

Conclusions

Fractionation factor calculations showed fewer anomalies when media to cell calculations rather than media to media calculations were used.

The isotopic fractionation between media and cells (+1.3 to -20.8 ‰) of *Chlorobium vibrioforme* grown in batch culture increased with the age of the culture.

C. vibrioforme cells which possessed the reverse TCA cycle fractionated carbon less than did bacteria having the Calvin cycle. The results from *Prosthecochloris aestuarii* supported this conclusion, and suggested that this may be a generalization for the Chlorobiaceae.

FAST ATOM BOMBARDMENT SPECTROMETRY OF *ECTOTHIORHODOSPIRA*

(Jaap J. Boon)

Fast atom bombardment mass spectrometry (FAB-MS) is a relatively novel technique to desorb polar organic molecules in the ion source of a mass spectrometer. In the past, the application of mass spectrometry to polar molecules was severely limited, due to the inability to produce the corresponding ions in the gas phase. Prior to volatilization, the molecules were decomposed by thermolytic processes.

FAB-MS has been applied to the analysis of inorganic salts, organic salts, nucleoside phosphates, underivatized peptides *etc.* (Williams *et al.*, 1981, Burlingame *et al.*, 1982). No information was available concerning the analysis of complex macromolecular mixtures as represented by whole cells. Analysis of a few bacterial samples was undertaken in the hope of finding a relatively simple way to characterize intracellular solutes. During this summer program a few hours of instrument time were available on a Kratos mass spectroscope 50 equipped with a Xenon-FAB source at the National Institute of Health Mass Spectrometry resource in San Francisco. Samples from *Ectothiorhodospira halophila* (whole cells, water wash and residue), *Chlorobium* (freeze dried) and Solar Lake cyanobacterial mat water (Sinai Desert) were analysed. Because betaine ($C_5H_{11}O_2N$) has been recently found as a major compatible solute against high salinities in the environment, the samples of *E. halophila* were especially interesting (Galinski and Trueper, 1982).

Principles of the Volatilization Method

Accelerated xenon ions were first produced from xenon atoms (kinetic energy of 8 Kev.) of high translational energy (ion beam current 40 μamps). They were converted to xenon atoms by charge exchange in the ion source of the mass

spectrometer. The 8 Kev beam was then impacted on the sample, mixed with glycerol, and applied to a copper probe tip. The operating pressure in the source was 10^{-6} torr. (The mechanisms of generation and desorption of ions is not yet completely understood.)

Molecular weight information is usually obtained as $(MH)^+$ ions in positive spectra. During bombardment with the atoms, charge transfer took place in the glycerol matrix, leading to protonated molecular ions. The desorbed positive ions were subsequently accelerated, focused and separated in the double focusing magnetic sector and then detected by an electronmultiplier. Ion intensities were plotted in the mass range from 600-35 at 300 sec/decade using UV sensitive chart paper.

Sample Analysis

Ectothiorhodospira halophila was grown on the medium (20 percent NaCl) of Imhoff *et al.* (1981). Whole cells were spun down at 10,000 xg in a Sorvall RC2 refrigerated centrifuge. About half of the harvested amount of these cells was washed with distilled water in an attempt to preferentially extract the betaine by partial lysis (Trueper, personal communication). After centrifugation, a clear, viscous, yellowish supernatant was obtained. Partial decolorization of the pellet suggested lysis of cells (this sample was labelled "residue" after a water wash).

The *Chlorobium vibrioforme* material was an aliquot of freeze dried cells set aside from the *delta* ^{13}C experiments. The cyanobacterial mat sample which had been stored for 13 months at -8 °C was taken from the 0.5-2 mm *Microcoleus* mat in shallow water from Solar Lake (Sinai Desert).

Analytical Results of the Samples

Figures II-6 to II-10 are the FAB mass spectra from *E. halophila* cells, water wash and residual material after water washing, *Chlorobium* cells (freeze dried) and cyanobacterial mat water. In general, most of the mass peaks seem to be generated from the glycerol matrix, except for a few major peaks in *E. halophila* samples. Table II-IX lists a number of assignments for mass peaks derived from the matrix material found in these spectra. The spectrum of the freeze dried *Chlorobium* was fully dominated by (glycerol) H^+ monomers, dimers and trimers *i.e.* peaks with masses at: 93, 185 and 277.

Several peaks in this spectrum point to ions of water (H_2O=18 a.m.v.) from glycerol $_nH^+$ e.g. 259, 241, 223, 167, 149, 131, 75, 57.

Fig. II-6 Fast Atom Bombardment Mass Spectrum of *Ectothiorhodospira halophila* (whole cells)

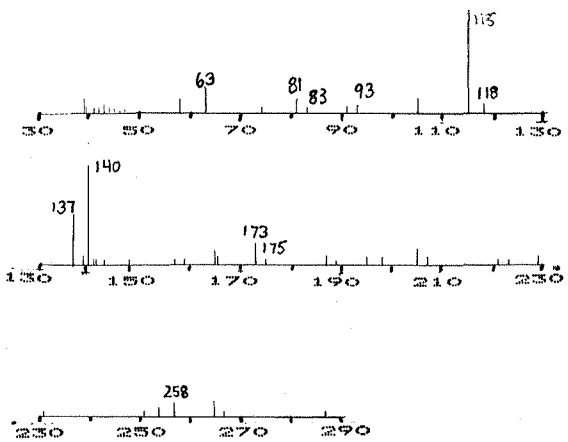


Fig. II-7 FAB—MS of *E. halophila* (partial lysis of cells)

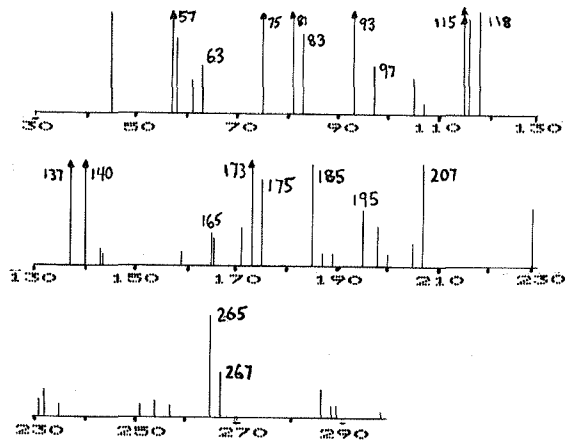


Fig. II-8

FAB-MS of *E. halophila* (after water washing)

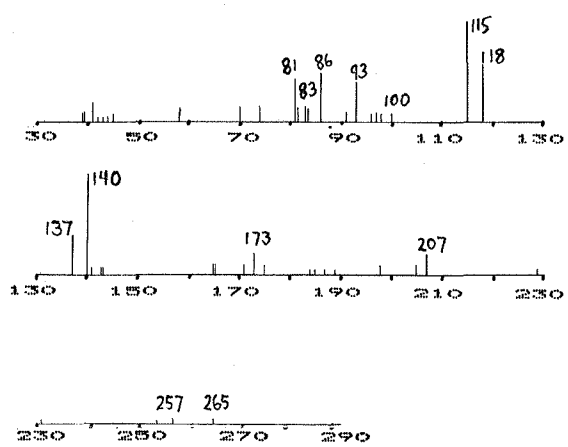


Fig. II-9

FAB—MS of freeze dried *Chlorobium*

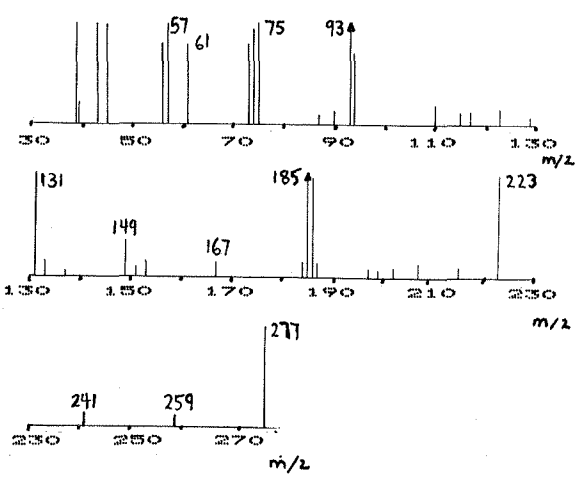


Fig. II-10 FAB-MS of Soda Lake top mat

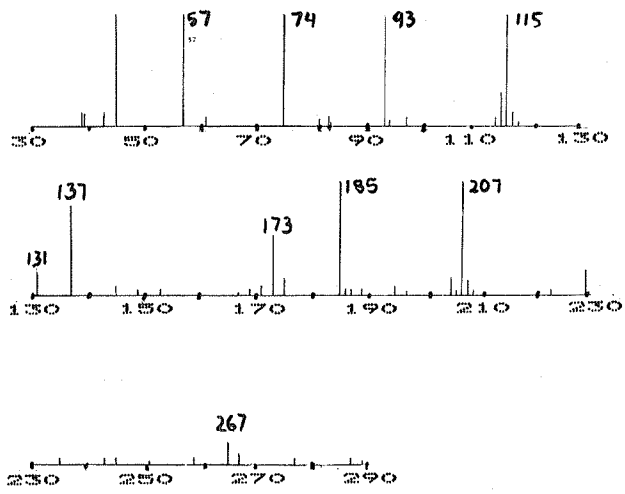


Table II-IX Proposed assignment of ions due to matrix effects in FAB-MS spectra

m/2	
57	(glycerol)H - 2 x H_2O
61	low eV fragmentation product of glycerol
75	(glycerol)H - 1 x H_2O
81	$(NaCl)NA^+$ (^{35}Cl)
83	$(NaCl)Na^+$ (^{37}Cl)
93	$(glycerol)H^+$
115	$(glycerol)Na^+$
131	$(glycerol)_2H^+ - 3H_2O$
137	$(glycerol)NaNa^+$
149	$(glycerol)_2H^+ - 2H_2O$
167	$(glycerol)_2H^+ - 1H_2O$
173	$(glycerol)(NaCL)Na^+$ (^{35}CL)
175	$(glycerol)(NaCl)Na^+$ (^{37}Cl)
185	$(glycerol)_2H^+$
195	$(glycerol)(NaCl)NaNa^+$ (^{35}CL)
197	$(glycerol)(NaCl)NaNa^+$ (^{37}CL)
207	$(glycerol)_2Na^+$
223	$(glycerol)_3H^+ - 3H_2O$
229	$(glycerol)_2NaNa^+$
241	$(glycerol)_3H^+ - 2H_2O$
259	$(glycerol)_3H^+ - 1H_2)$
265	$(glycerol)_2(NaCl)Na^+$ (^{35}Cl)
267	$(glycerol)_2(NaCl)Na^+$ (^{37}Cl)
277	$(glycerol)_3H^+$
287	$(glycerol)_2(NaCl)NaNa^+$

These latter ions were not observed in the wet cells from saline media. Instead, sodium was seen as a major cation in glycerol complexes. The mass charge 115, interpreted as sodium cationized glycerol (glycerol.Na^+), was the base peak in these spectra. Several other cationized species were assigned *e.g.* (glycerol)$_n$NaNa$^+$ (137, 220), (glycerol) Na^+ (115,207); (glycerol)(NaCl)Na^+ (81, 83, 173, 175, 265, 267).

The possibility of (glycerol)$_n$(NaCl)Na^+ complexes is proposed because of the presence of mass peaks with ratios pointing to chlorine (natural abundance of 75 percent ^{35}Cl and 25 percent ^{37}Cl). The relative abundances of the various cationized glycerol species appeared to be dependent on the concentration of the NaCl in the glycerol matrix. This is a phenomenon to be investigated further.

Several mass peaks in the *E. halophila* spectra could not be explained as matrix effects. Cells, water wash and residue showed a major mass peak at mass 2, 118 and 140. These peaks were interpreted as (betaine)H^+ and (betaine)Na^+. Confirmation of these identifications awaits the analysis of the standard. In the whole cells, the peaks at mass 140 and 115 were of equal abundance, while most glycerol matrix peaks are greatly suppressed. Such a result is typical when an organic compound is desorbed efficiently.

Other mass peaks in the spectra of *E. halophila*, not due to the glycerol matrix, were the mass 2, 63, 74, 86, 97, 100, 105, 141 (doublet) 143, 148, 157, 159, 165, (doublet), 187, 189, 198, 221, 231, 232, 251, 254, and 257. At higher masses (up to mass 21000), small signals were observed in the whole cell spectrum of *E. halophila*. Interpretation of most of these mass peaks is difficult at this stage and would require an extensive survey with standards.

The cyanobacterial mat sample (Fig. II-10) showed mostly cationized glycerol species. It is likely that the sample to glycerol ratio was not correct in this analysis. No betaine mass peaks were detected, although the presence of betaine was inferred from the intensity of trimethylamine in pyrodysin mass spectra of these mats (Boon *et al.*, 1981).

Conclusions

Bacterial cells can be introduced into a FAB mass spectrometer by dissolution in glycerol matrix. Wet cells seem to produce better signals than freeze dried cells.

Major ionic organic molecules can be desorbed from wet bacterial cells in FAB-MS mode.

Surface chemical phenomena lead to cationized species which may be used to determine alkali metal composition.

FAB-MS is a relatively simple way to demonstrate the presence of betaine in bacterial cells. The method shows promise for rapid surveying of a number of halotolerant and halophilic bacteria.

THE ABSORBANCE SPECTRA OF SEVEN DIFFERENT PHOTOTROPHIC BACTERIA

(Karinlee Kneller)

Introduction

Several methods were widely employed to determine the absorption spectra of eukaryotic and prokaryotic chlorophylls, carotenoids and auxiliary pigments. This involved use of whole cells, placement of whole cells in 50 percent sucrose, 100 percent methanol extraction, and 90 percent aqueous acetone extraction. The characters of the absorbance spectra obtained from these methods differed. Whole cells, with or without 50 percent sucrose, contained cell material which tended to scatter light, thereby reducing or masking the maximal absorbance of the pigments. When organically extracted pigments were separated from the cells less light was scattered, and the positions of the maximal absorbances were shifted.

This study attempted to compare the relative efficencies of three of the different methods above to determine which was best suited to obtaining spectra from phototrophic bacteria.

Methods and Materials

Pure cultures of *Chlorobium vibrioforme*, *Chromatium vinosum*, *Chromatium warmingii* and *Ectothiorhodospira shaposhnikovii* were grown according to the methods described in Appendix II. In addition, a pure culture of *Chloroflexus aurantiacus* obtained from Dr. Beverly Pierson was maintained according to the methods described by Castenholz and Pierson (1981). Two enrichment cultures of marine organisms, *Prosthecochloris aestuarii* and *Rhodopseudomonas*, were also grown.

Fig.II-11 Absorbance Spectra of *Chlorobium vibrioforme*

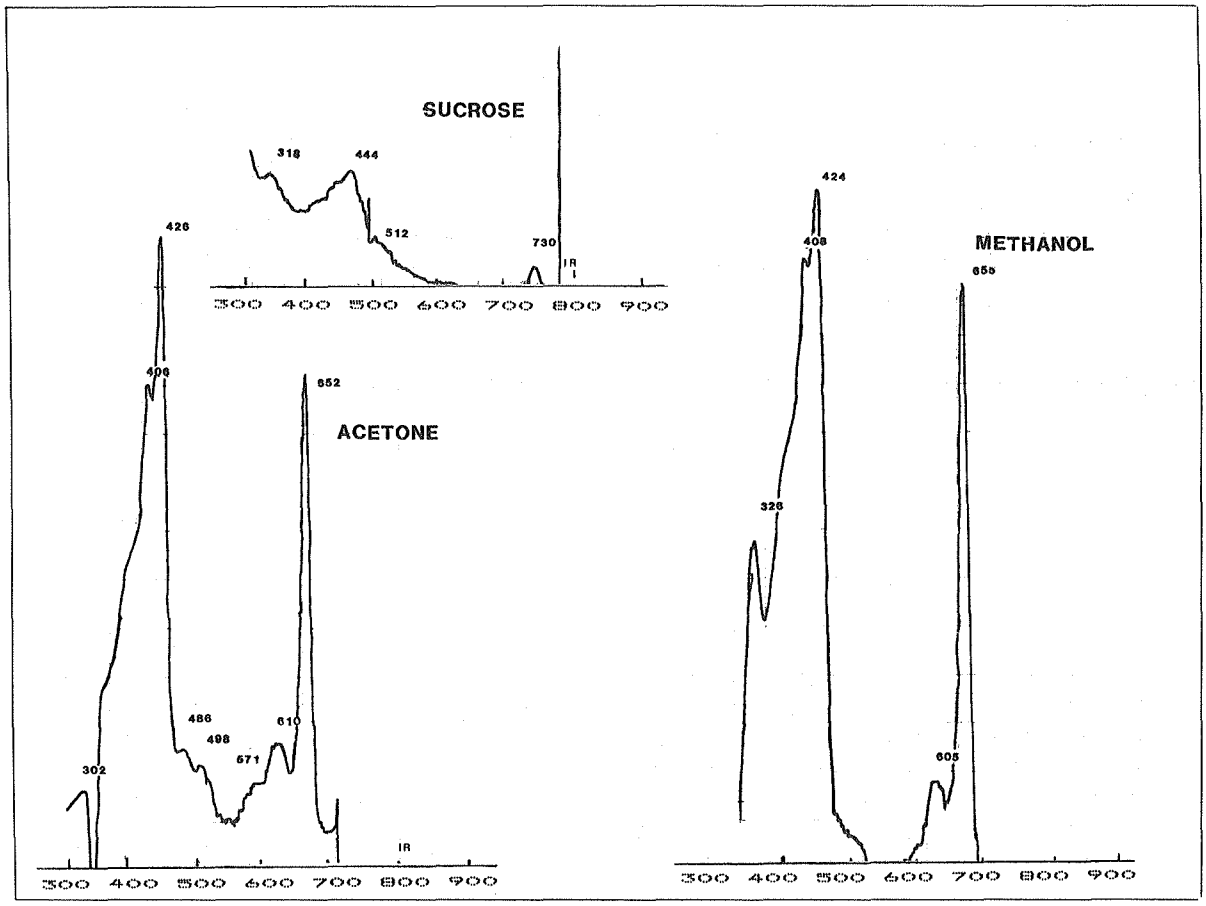


Fig. II-12 Absorbance Spectra of *Chromatium vinosum*

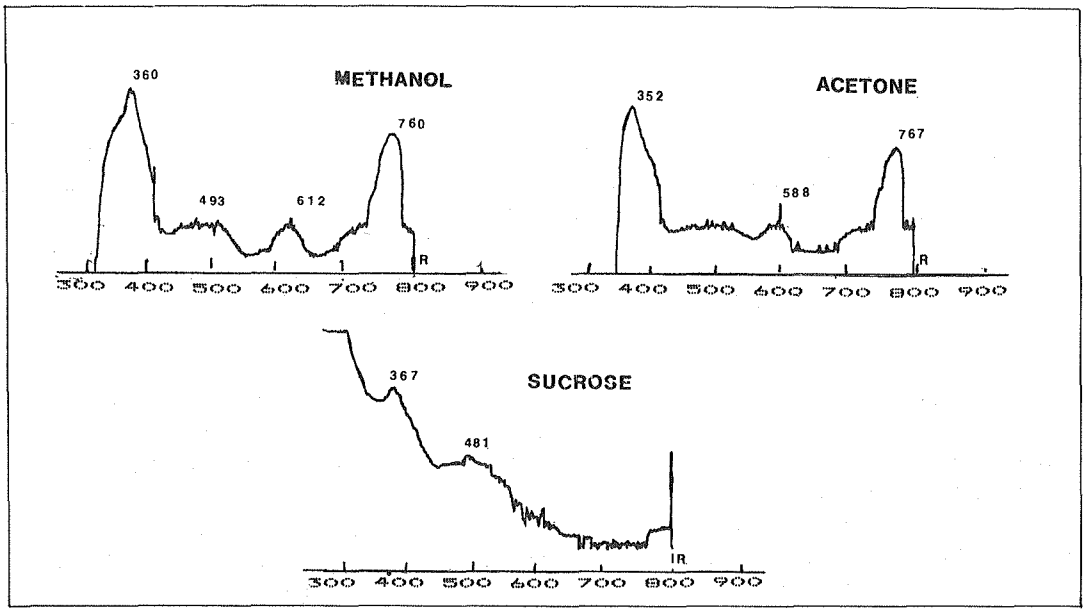


Fig. II-13 **Absorbance Spectra of *Chromatium warmingii***

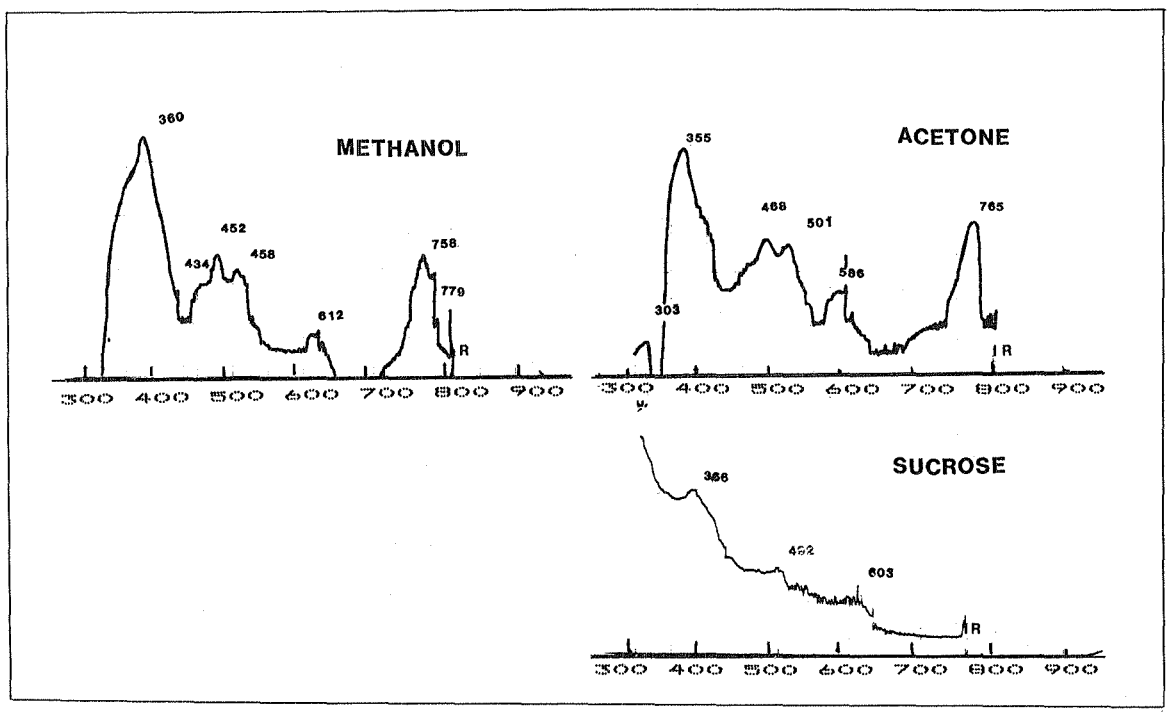


Fig. II-14 **Absorbance Spectra of *Ectothiorhodospira shaposhnikovii***

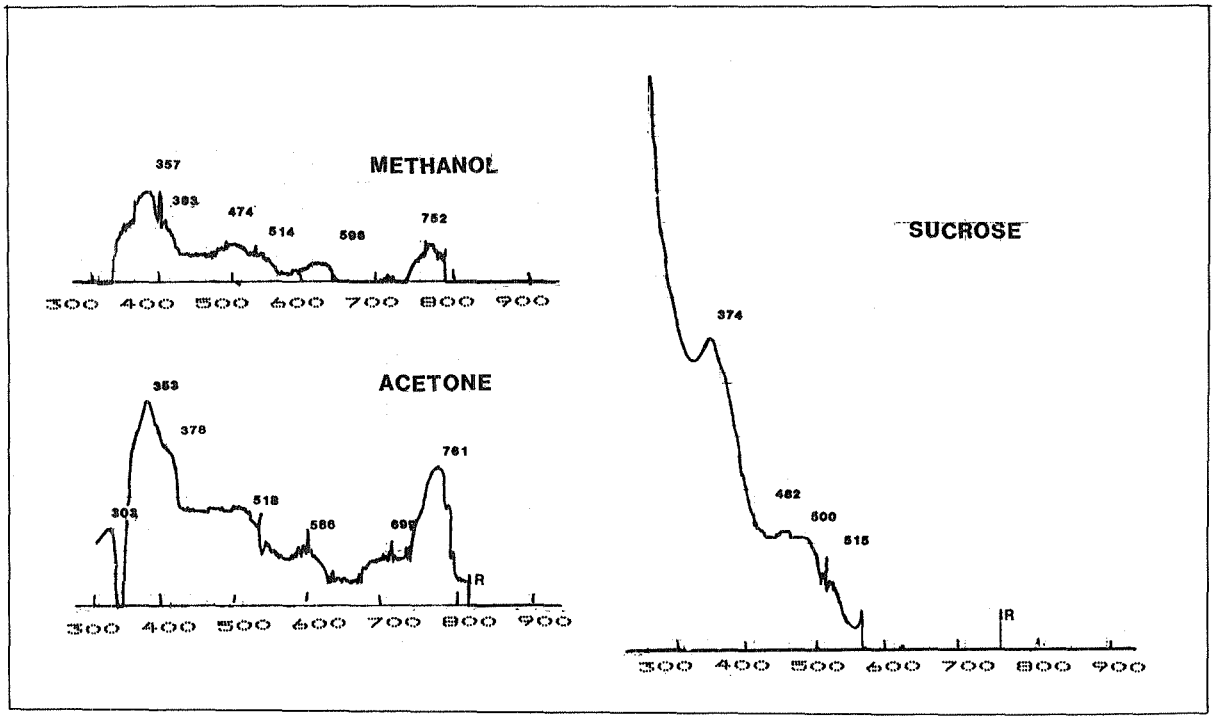


Fig. II-15 Absorbance Spectra of *Chloroflexus sp.*

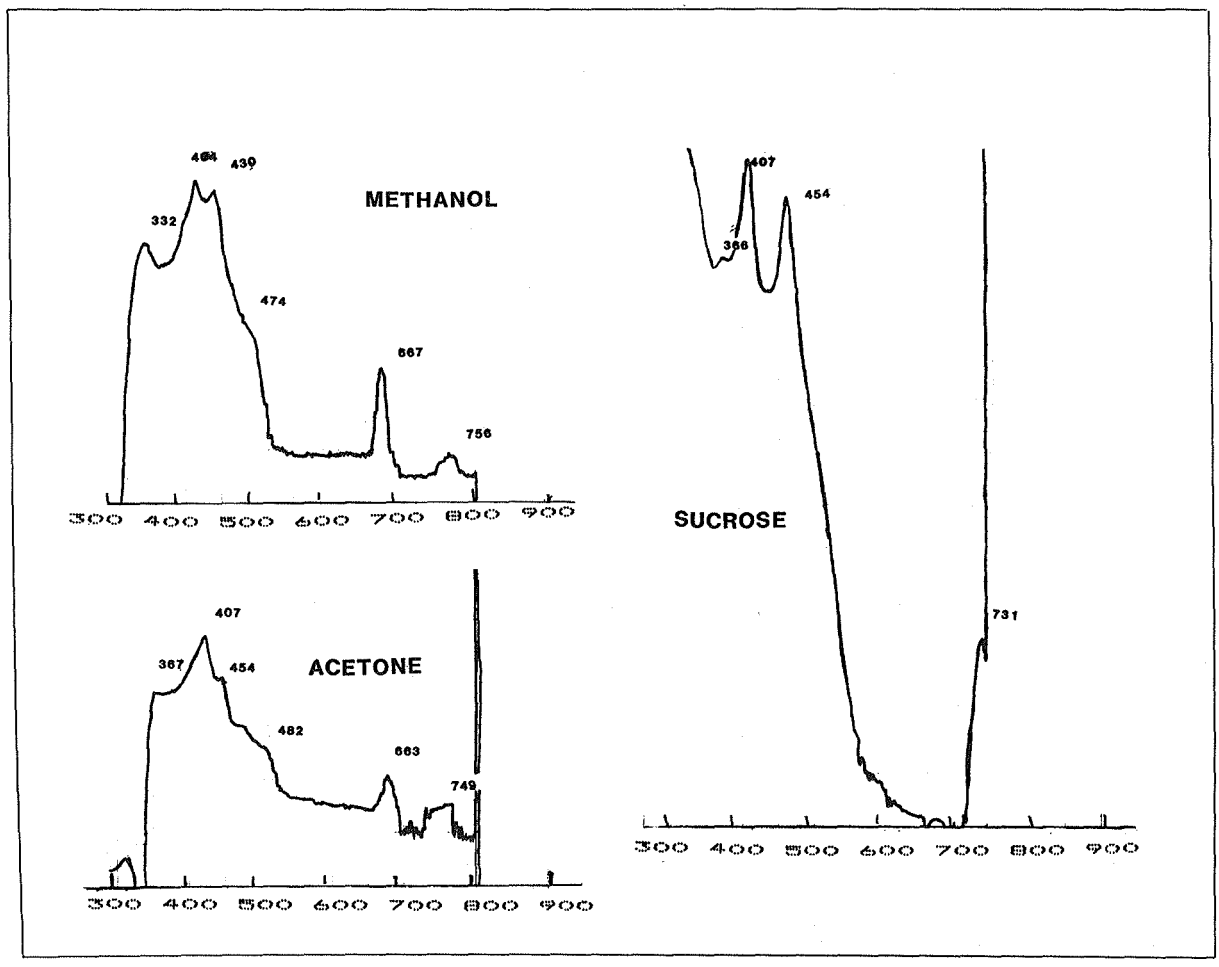


Fig. II-16 Absorbance Spectra of *Prosthecochloris aestuarii*

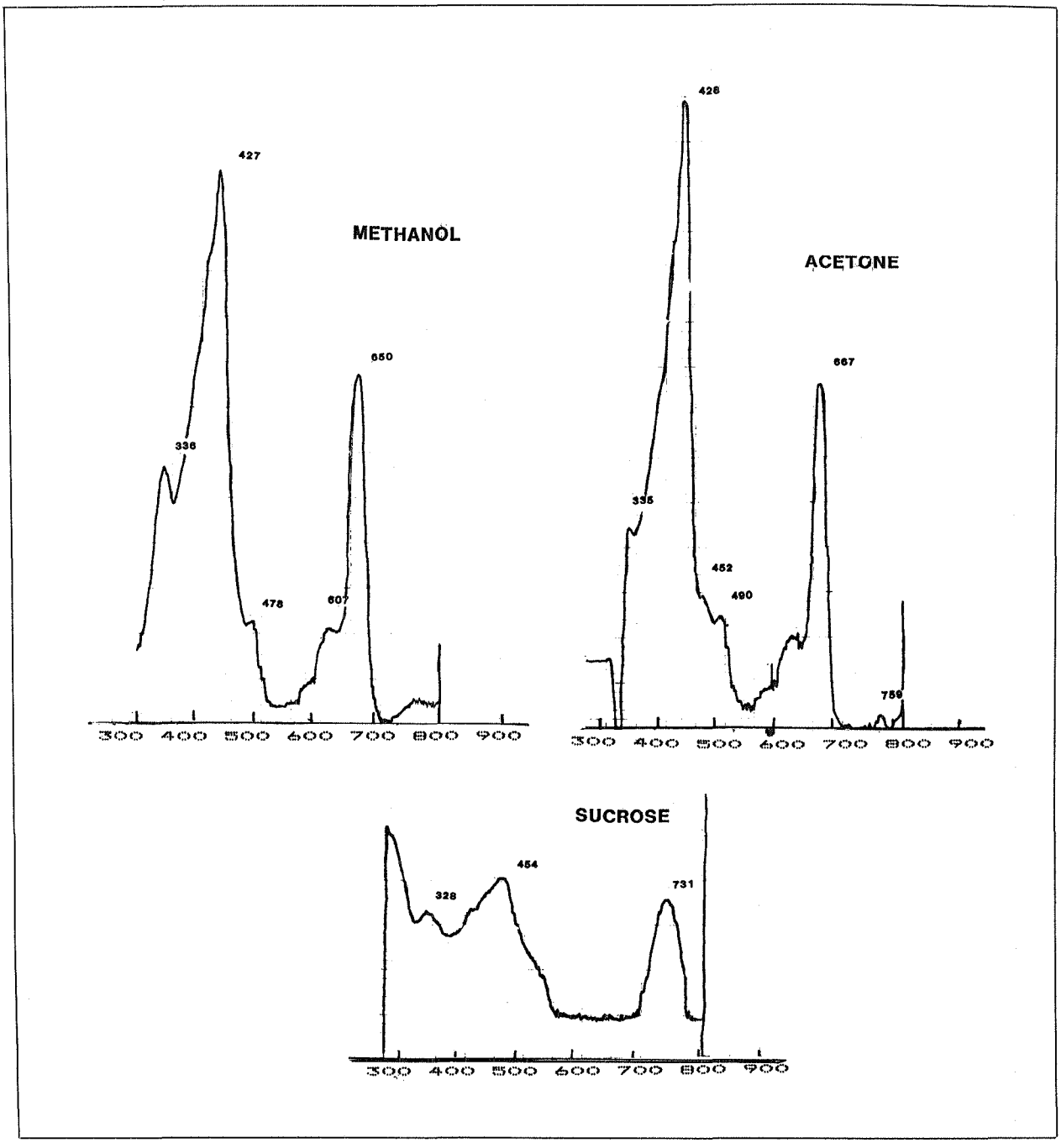
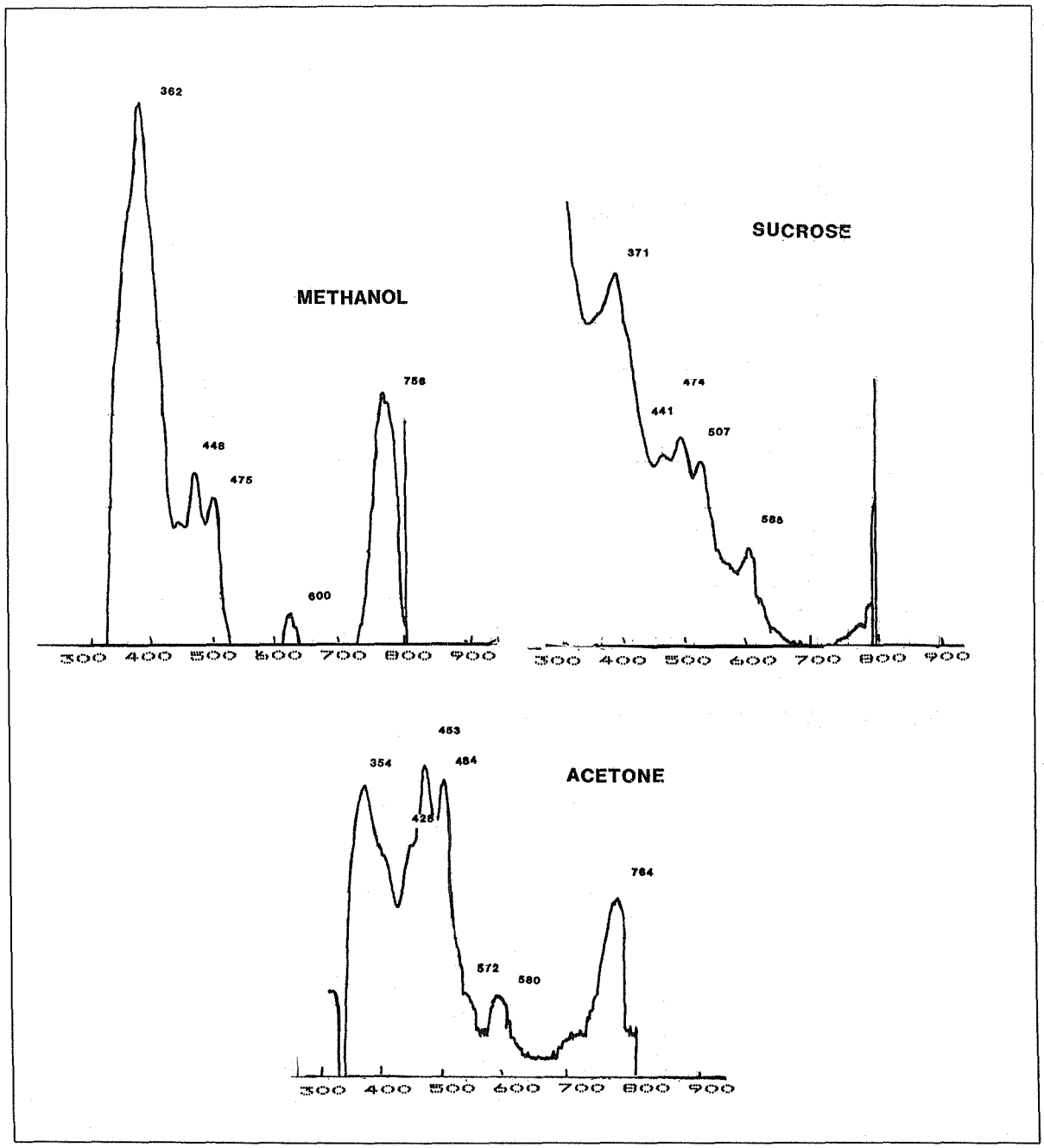


Fig. II-17 Absorbance Spectra of *Rhodopseudomonas sp.*



The volume of culture used for the absorption spectra was determined by the cell density of the cultures at the time of sampling. 1 ml aliquots were taken from the *C. aurantius* culture; 2 ml aliquots were taken from each of the *C. vibrioforme*, *Rhodopseudomonas*, *E. shaposhnikovii* and *C. vinosum* cultures; and 3 ml aliquots were taken from the *P. aestuarii* and *C. warmingii* cultures. For the spectra taken in sucrose, each aliquot of cell culture was transferred to a 15 ml Corex centrifuge tube containing 5 g of sucrose. Distilled water was added to bring the total volume of fluid to 5 ml. After dissolution in the sucrose, the samples were centrifuged (Sorvall RC-2) at 10,000 xg for 10 min. The supernatant was then decanted into test tubes, from which 2 ml aliquots were removed and transferred to 3 ml quartz spectrophotometric cuvettes. The same aliquots of culture were centrifuged as above for the analysis in methanol. To the pellet, 5 ml of anhydrous methanol were added and allowed to stand for 5 minutes in the dark at 4°C and then recentrifuged. The supernatant was decanted and reserved for spectrophotometric analysis. Sample preparation for the spectral analysis in acetone was the same as for methanol, however, 5 ml of 90 percent aqueous acetone buffered with ammonium hydroxide (to pH 10) was added to the pellet.

All spectra were determined with a Carey-14 spectrophotometer. Each absorbance spectrum was determined from 1000 nm to 300 nm (at 50 angstroms/sec) on a 0-1 absorbance scale. The shift from the IR wavelength range to the visible wavelength range was at approximately 800 nm.

Results and Discussion

Figures II-11 through II-17b are the absorbance spectra for the seven organisms in each of the three extract solutions. Note that in all the sucrose absorbance spectra the maximal peaks are poorly defined, and the long wave bacteriochlorophyll peak (740-810 nm) is missing. The sucrose absorbance spectrum for *P. aestuarii* had the best resolution. This may reflect the fact that the *P. aestuarii*, as the youngest culture, had the smallest amount of light-scattering cell debris.

Both the methanol- and the acetone-extracted samples gave absorbance spectra with well-defined and highly resolved peaks of maximal absorbance. In addition, both types of spectra had the long wave bacteriochlorophyll maxima. Though the positions of the maximal absorbance peaks are comparable, they were shifted in

an unpredictable manner.

Consequently, in pure cultures of low cell density, the use of sucrose analysis, recommended by Trueper and Pfennig (1981), was sufficient to distinquish and identify species. However, under high cell density or in field samples of high diversity either the methanol- or acetone-extraction procedures are recommended to characterize, distinguish and identify the bacteriochlorophylls.

BACTERIAL POPULATIONS AND ATP DETERMINATION

(Deborah B. Craven)

Big Soda Lake, Nevada, is an alkaline, moderately hypersaline lake, that shows a seasonal bloom of phototrophic sulfur bacteria at the chemocline (Oremland *et al.* 1982, Cloen *et al.* 1982). The organisms causing this bloom have been isolated and identified during this research program (see page 65) as *Ectothiorhodospira vacuolata* (Imhoff *et al.* 1981). In this project, the ATP present in the microorganisms was compared with that in living cells in the water column.

Methods

The content of 1 vial FLE-50 (Sigma) was hydrated in 5 ml distilled water for 10 hours. Then 20 ml of 0.1M potassium arsenate buffer, pH 7.4, 20 ml 0.04M $MgSO_4$ and D-luciferin (10 μg/ml FLE-50 diluted volume) were added. This mixture was allowed to sit another 10 hours, and then filtered through a 0.2 μm Millipore filter just before use.

ATP was extracted from the water samples by filtering the cell material onto 0.2 μm Gelman filters which were then immediately placed in test tubes containing 5 ml 60 m*M* potassium-phosphate buffer, pH 7.4, in a boiling water bath. The tubes were boiled, loosely capped, for about 5 min, then cooled and frozen. The ATP assays, performed at room temperature, were calibrated against an ATP standard curve in 60 m*M* phosphate buffer. One ml FLE-50 was used as assay mixture for 0.2 ml samples. Living cell counts were prepared in agar shake dilution medium of Trueper and Imhoff, 1981 (see Appendix II).

Results and Discussion

The results are shown in Table II-X and Figure II-18. The distinct ATP maximum at 22-25 meter depth agreed absolutely with the bacteriochlorophyll *a* and the absorbance maxima reported for July, 1982 (Cloen *et al.*, 1982). Unfortunately, the cell counts could not be performed before the end of the program.

Fig. II-18
ATP Distribution Curve in Big Soda Lake Samples from July, 1982

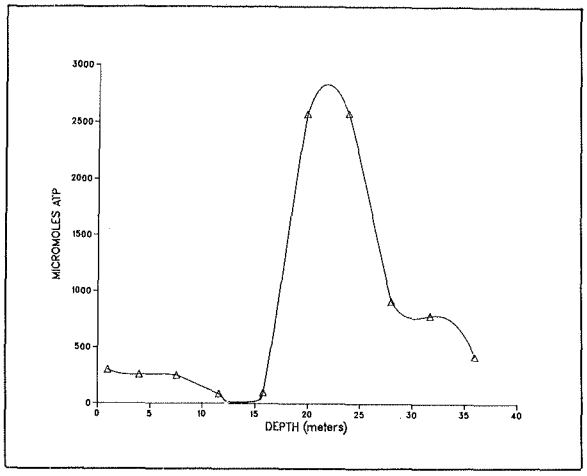


Table II-X ATP Determination in Big Soda Lake Water Samples

Depth, m	ATP nanograms per ml	filtered volume (ml)	nanograms per liter^{-1}
1	0.928	16.0	290.0
5	0.857	16.0	268.0
10	0.771	15.0	257.0
15	0.286	15.5	92.5
20	0.714	15.2	235.0
22	8.780	17.0	2582.5
25	7.930	15.3	2591.5
30	2.860	15.8	905.0
35	2.570	16.8	765.0
40	1.360	16.5	412.0

SUMMARY

(H. G. Trueper)

The species occurring in the field samples were tentatively identified by light microscopy. Using enrichment cultures and direct inoculation techniques, more than seven pure cultures of

Rhodospirillaceae, Chromatiaceae and Chlorobiaceae were isolated. Several of these, and several pure cultures that had been brought to Santa Clara were analyzed for ^{13}C fractionation rates during phototrophic CO_2 fixation. In the case of *Chlorobium*, these experiments were combined with growth measurements. Valuable data were thus obtained for Chlorobiaceae and Chromatiaceae. The isotope fractionation between the cells and the medium increased with the age of the culture. The amount of ^{12}C fractionation was measured for *Chlorobium*, several species of *Chromatium*, *Ectothiorhodospira*, and *Prosthecochloris*. The green bacteria, in general, are less enriched in ^{12}C than the purples.

Samples of *E. halophila* were subjected to fast atom bombardment mass spectrometry. The method promises to be a useful tool for the detection of metabolites and cell constituents such as betaine and perhaps even as a "fingerprinting" method in bacterial taxonomy.

A study of different methods to obtain absorption spectra of "whole cells" and cellular photopigments of pure cultures of purple and green bacteria showed that different application ranges have to be taken into consideration when these methods are used in field work. Sucrose is an adequate solvent for pure cultures of low cell density whereas methanol or acetone extractions are recommended for high density, mixed cultures because they yield well defined, highly resolved bacteriochlorophyll peaks.

An ATP profile measured in the water column of an alkaline salt lake coincided optimally with the distribution of bacteriochlorophyll *a*. The organisms responible for the purple layer in the lake were isolated and identified as *Ectothiorhodospira vacuolata*.

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APPENDIX II
PHOTOTROPHIC BACTERIA MEDIA AND TECHNIQUES

Enrichment Medium for Rhodospirillaceae

Prepare the medium solution with distilled water:

KH_2PO_4	0.5 g/l
$MgSO_4.7H_2O$	0.2 g/l
NaCl	0.4 g/l
$CaCl_2.2H_2O$	0.05 g/l
Organic carbon compound	1.0 g/l
Yeast extract	0.2 g/l
Fe-citrate solution (0.1 g/100 ml)	5 ml/l
Trace element solution SL 7	1 ml/l
Cyanocobalamin (vitamin B12) solution (1.0 mg/100 ml)	1 ml/l

The composition of the trace element solution SL 7 is as follows:

Distilled water	1 liter
HCl (25 percent)	1 ml
$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	100 mg
H_3BO_3	60 mg
$CoCl_2.4H_2O$	200 mg
$CuCl_2.2H_2O$	20 mg
$NiCl_2.6H_2O$	20 mg
$NaMoO_4.2H_2O$	40 mg

Dissolve the components of the medium in the order given. Adjust the pH to 6.8, when dicarboxylic acids are the substrates, or to 7.3 when fatty acids are used. For the enrichment of *Rhodopseudomonas acidophila* and *Rhodomicrobium vannielii*, the initial pH should be 5.2-5.5 (5.5-6.0 for agar plates incubated under N_2/CO_2). Liquid medium is most favorably sterilized in 50 ml metal screw-cap bottles with autoclavable rubber seals, which later serve as culture vessels. Fill and autoclave the bottles with about 45 ml of medium for 45 minutes at 120°C with loose screw caps in a metal bucket. Close the bucket with a felt cloth or synthetic foam. After cooling to room temperature, close the bottles tightly. They can be stored for several months. Fill them with medium prior to inoculation, leaving a small air bubble.

When fatty acids or alcohols are the substrates, bicarbonate is necessary for growth. It can be added to each bottle of sterile medium from a filter-sterilized solution of 5 percent sodium bicarbonate to give a final concentration of 0.2 percent.

Direct Isolation on Agar Plates

This method is similar to the one used to enumerate heterotrophic bacteria. The medium is only moderately selective, but the incubation conditions are highly selective.

Use the medium as described and supplement it with 1.5 percent agar; succinate is recommended as the carbon source. Pour about 20 ml of the sterile, liquified agar medium into each standard, plastic Petri dish.

One of three different procedures of inoculation should be used, depending on the number of cells in a sample. When a high number of purple phototrophic bacteria is expected (above 100 cells/ml), the streak-plate method can be applied: drop 0.2 ml of the sample or of one of its dilutions onto the agar and distribute it using a Drigalski spatula or an inoculation loop. Place the plates in anaerobic GasPak jars (Becton, Dickinson and Co.) (The jar operates with chemicals that produce hydrogen and carbon dioxide; part of the hydrogen reacts with the oxygen in the jar in the presence of a catalyst). Incubate the jars in incandescent white light of low intensity (200 - 1,000 lux).

After 6-10 days, the plates are ready for examination. The colonies of purple nonsulfur bacteria are always clearly recognizable by their intense coloration.

Culture Media for Chromatiaceae and Chlorobiaceae

The composition and preparation of two different culture media are described below for the cultivation of green and purple sulfur bacteria. Medium 1 is suitable for most green and purple bacteria presently in laboratory culture, including those species that are most difficult to grow (e.g., *Thiopedia rosea*, *Thiospirillum jenense*, *Chromatium okenii*, and *Thiodictyon elegans*). With minor modifications, this culture medium was published by Pfennig (1965), Pfennig and Lippert (1966), Trueper (1970), and van Niel (1971). Medium 2 (Biebl and Pfennig, 1978) is simpler to prepare and can be successfully used for the cultivation of the most common green and purple sulfur bacteria.

Medium 1 for Cultivation of Green and Purple Sulfur Bacteria

(Pfennig's medium)

Prepare the medium in a 5-liter bottle with four openings at the top. There are two openings for tubes in the central, silicon rubber stopper at the top. One is a short, gas-inlet tube with a sterile cotton filter. The other is an outlet tube for medium. The outlet tube reaches the bottom of the vessel at one end and has a silicon rubber tube with a pinch cock and a bell for aseptic dispensing of the medium into bottles at the other end. The other two openings in the bottle have gas-tight screw caps. One of these openings is for the addition of sterile solutions and the other serves as a gas outlet.

The composition of Medium 1 is given for a total of 5 liters of culture medium.

Solution 1:

Distilled water	4,000 ml
KH_2PO_4	1.7 g
NH_4Cl	1.7 g
KCl	1.7 g
$MgSO_4.7H_2O$	2.5 g
$CaCl_2.2H_2O$	1.25 g

For enrichment cultures, or pure cultures from marine or estuarine habitats, add 100 g NaCl to Solution 1 and increase the $MgSO_4.7H_2O$ to 15 g.

Autoclave Solution 1 with a Teflon-coated magnetic bar for 45 min at 121° C in the 5-liter bottle. Cool it to room temperature in an N_2 atmosphere with a positive pressure of 0.05-0.1 atm (a manometer for low pressures is required). Then saturate the cold medium with CO_2. Saturation is achieved by magnetically stirring for 30 min in a CO_2 atmosphere of 0.05-0.2 atm. Then add the following sterile solutions 2 through 6 through one of the screw-cap openings while magnetically stirring the medium against a stream of either N_2 gas or, better, a mixture of 95 percent N_2 and 5 percent CO_2.

Solution 2: Distilled water 860 ml

After autoclaving in a cotton-stoppered Erlenmeyer flask, cool the hot water to room temperature in an atmosphere of N_2 in an anaerobic jar.

Solution 3: 5 ml Vitamin B_{12} solution (2 mg/100 ml)

Solution 4: Prepare the following autoclaved stock solution:

Distilled water	993 ml
HCl (25 percent)	6.5 ml
$FeCl_2.7H_2O$	1.5 g
H_3BO_3	62 mg
$MnCl_2.4H_2O$	100 mg
$CoCl_2.6H_2O$	24 mg
$ZnCl_2$	70 mg
$NiCl_2.6H_2O$	24 mg
$CuCl_2.2H_2O$	17 mg
$Na_2MoO_4.2H_2O$	36 mg

Solution 5: Sterile 7.5 percent Na HCO_3 solution 100 ml

Flush the solution until saturated with CO_2 on a magnetic stirrer and filter-sterilize it into sterile, gas-tight, 100 ml screw-cap bottles.

Solution 6: Sodium Sulfide Medium

Add 20 ml of a sterile, 10 percent $Na_2S.9H_2O$ solution to the medium for purple sulfur bacteria, or 30 ml to the medium for green sulfur bacteria. Prepare the

sulfide solution in a screw-cap bottle. After replacing the air with N_2, tightly close and autoclave the bottle.

After combining and mixing solutions 1 through 6, adjust the pH of the medium with sterile HCl or Na_2CO_3 solution (2 *M* each) to pH 6.8 for green sulfur bacteria or to 7.3 for purple sulfur bacteria. Then distribute the medium aseptically through the outlet tube into sterile, 100 ml bottles with metal caps and autoclavable rubber seals. Use the positive gas pressure (0.05-0.1 atm) of the N_2/CO_2 gas mixture. Leave a small air bubble in each bottle to meet possible pressure changes. The tightly sealed, screw-cap bottles can be stored for several weeks or months in the dark. During the first 24 hours, the iron of the medium precipitates in the form of black flecks. No other sediment should arise in the otherwise clear medium.

Supplemental Solutions

With the amount of $Na_2S \cdot 9H_2O$ initially added to Medium 1 (higher initial amounts may be inhibiting for some species), only very limited growth can be expected. When sulfide and sulfur are photooxidized, the bacteria stop growing and are damaged by further illumination. In order to keep the cultures growing and to obtain high cell yields it is necessary to feed the cultures several times with sterile, partially neutralized sulfide solution, which is prepared from Solution 7.

Solution 7: Partially Neutralized Sulfide Solution of Feeding Cultures of Green and Purple Sulfur Bacteria

Distilled water	100 ml
$Na_2S \cdot 9H_2O$	3 g

Prepare the solution in a 250 ml screw-cap bottle. After replacement of the air by N_2 tightly close and autoclave the bottle.

To prepare the partially neutralized sterile feeding solution, add a measured amount of sterile Solution 7 to a sterile Erlenmeyer flask with a magnetic bar. Bring the solution to about pH 8.0 by dropwise addition of sterile 2 *M* H_2SO_4 on a magnetic stirrer. If too much acid is added, the sulfide solution becomes turbid due to precipitation of elemental sulfur. Use the partially neutralized solution immediately for the feeding of 100 ml bottle cultures. Depending on the population density, use

1-2 ml for Chromatiaceae and 2-3 ml for Chlorobiaceae. Before the addition, aseptically remove an equivalent amount of culture medium from the bottle culture.

Solution 8: Thiosulfate Solution for Cultivation of Green and Purple Sulfur Bacteria

Cultures of green and purple sulfur bacteria that can use thiosulfate as an electron donor can be supplemented with 0.1 percent of this compound from stock solution.

Distilled water	95 ml
$Na_2S_2O_3.5H_2O$	10 g

Prepare and autoclave the solution in a 200 ml screw-cap bottle. Then add 1 ml aseptically to 100 ml of culture medium.

Growth yields of green and purple sulfur bacteria can be increased by the addition of acetate as a readily assimilated carbon source; 0.03 percent or 0.05 percent acetate is regularly added to agar shake dilution cultures. Acetate can be added to liquid cultures only when they are free of purple nonsulfur bacteria. The ammonium and magnesium salts of acetate are used to avoid strong pH changes during growth.

Solution 9: Acetate Solution for Cultivation of Green and Purple Sulfur Bacteria

Distilled water	100 ml
Ammonium acetate	2.5 g
Magnesium acetate	2.5 g

Prepare and autoclave the solution in a 200 ml screw cap bottle. Application is 1 ml, added aseptically to 100 ml of culture medium.

Medium 2 for Cultivation of Green and Purple Sulfur Bacteria

(Biebl and Pfennig, 1978)

Prepare this medium in a 2-liter Erlenmeyer flask with an outlet near the bottom on one side. Connect a silicon rubber tube (about 30 cm long) with a pinch cock and a bell for aseptic

distribution of the medium into bottles to the outlet. Put a magnetic bar into the flask.

Solution 1: Phototrophic Bacterial Medium

Distilled water	950 ml
Solution 2 (SL 8)	1.0 ml
KH_2PO_4	1.0 g
NH_4Cl	0.5 g
$MgSO_4.7H_2O$	0.4 g
$CaCl_2.2H_2O$	0.05 g

For marine strains, add 20 g NaCl to Solution 1 and increase the $MgSO_4.7H_2O$ to 3 g. Autoclave the solution in the cotton-plugged, 2-liter Erlenmeyer flask.

Solution 2: Trace element solution (SL8)

Distilled water	1,000 ml
Ethylenediamine-tetraacetate-di-Na-salt	5.2 g
$FeCl_2.4H_2O$	1.5 g
$ZnCl_2$	70 mg
$MnCl_2.4H420$	100 mg
H_3BO_3	62 mg
$CoCl_2.6H_2O$	190 mg
$CuCl_2.2H_2O$	17 mg
$NiCl_2.6H_2O$	24 mg
$Na_2MoO_4.2H_2O$	36 mg

Dissolve the salts in the order given and store the solution in a refrigerator. Application is per liter of medium.

When the autoclaved Solution 1 (including Solution 2) is cold, add the following sterile Solutions 3 through 5 aseptically while magnetically stirring the medium:

Solution 3: Vitamin B12 Solution

1 ml (2 mg/100 ml; filter sterilize.

Solution 4: Sodium Bicarbonate Solution

40 ml of 5 percent $NaHCO_3$ in distilled water; filter sterilize.

Solution 5: Sodium Sulfide Solution

5 percent $Na_2S.9H_2O$

Prepare a freshly autoclaved 5 percent solution of $Na_2S.9H_2O$ in distilled water. Add 6 ml to the medium for purple sulfur bacteria, or 12 ml to medium for green sulfur bacteria.

After additions from Solutions 1 through 5, adjust the pH of the medium with sterile H_2SO_4 or Na_2CO_3 solution (2 *M* each) to pH 6.8 for green sulfur bacteria or to pH 7.3 for purple sulfur bacteria. Then dispense the medium aseptically into sterile, 50 or 100 ml bottles with metal screw caps containing autoclavable rubber seals. Leave a small air bubble in each bottle to meet possible pressure changes.

Medium 3 for haloalkaliphilic *Ectothiorhodospira* species

(Imhoff and Trueper, 1977)

Solution 1: Nutrient Medium

Dissolve the following components in distilled water to reach a final volume of 1 liter:

$CaCl_2.2H_2O$	0.05 g
$MgCl_2.6H_2O$	0.1 g
KH_2PO_4	0.5 g
NH_4Cl	0.8 g
Na_2SO_4	10.0 g
Na_2CO_3	6.0 g
$NaHCO_3$	14.0 g
NaCl	180.0 g
Na succinate	1.0 g
Yeast extract	0.5 g
$Na_2S.9H_2O$	1.0 g
Vitamin solution (VA)	1.0 ml

Solution 2: VA vitamin stock solution

Prepare stock solution:

Distilled water	100 ml
Biotin	10 mg
Nicotinamide	35 mg
Thiamine dichloride	30 mg
p-Aminobenzoic acid	20 mg
Pyridoxal chloride	10 mg
Ca pantothenate	10 mg
Vitamin B12	5 mg

Adjust the pH of the nutrient medium to 8.5-8.7 by adding 2 *M* Na_2CO_3. Sterilize the medium by membrane or Seitz filtration. Then add 1.0 ml of a sterile trace element solution ("SLA").

Solution 3: ("SLA")

Prepare as follows:

Distilled water	1,000 ml
$FeCl_2.4H_2O$	1.8 g
$CoCl_2.6H_2O$	250 mg
$NiCl_2.6H_2O$	10 mg
$CuCl_2.2H_2O$	19 mg
$MnCl_2.4H_2O$	70 mg
$ZnCl_2$	100 mg
H_3BO_3	500 mg
$NaMoO_4.H_2O$	30 mg
Na_2SeO_3	10 mg

Acidify the solution to pH 3.0 by adding 2N HCl. It may be stored for several months. For use in the medium, autoclave suitable portions before the addition. Distribute the medium to sterile screw-cap bottles that are filled completely. Leave a pea-sized air bubble to meet possible pressure changes. This medium may be used either directly for agar dilution series or for liquid enrichment culture.

Incubate cultures at 1,000-5,000 lux (approximately 100-500 foot candles) and temperatures of about 40-45° C.

Medium 4 for cultivation of *Ectothiorhodospira* sp.

Ectothiorhodospira shaposhnikovii and *E. mobilis* grow well in Pfennig's medium. Growth is markedly enhanced by the addition of organic acids, and sulfide may be replaced by thiosulfate.

During cultivation, liquid cultures on sulfide have to be monitored with respect to the disappearance of sulfide and elemental sulfur. Without a photosynthetic electron donor, cultures stop growing and will be damaged by further illumination. To avoid this and to obtain high biomass yields, it is necessary to feed such cultures with a sterilized solution of sulfide (Solution 7 of Pfennig's Medium). During photoautotrophic cultivation a depletion of carbonate diminishes the buffering capacity of the medium; therefore, we recommend a feeding solution containing per 100 ml of distilled water: $Na_2S.9H_2O$, 5.0 g and $NaHCO_3$, 10.0 g. This solution may be filter-sterilized or autoclaved and is fed to the cultures in amounts of 10 ml/liter.

Ectothiorhodospira halochloris and *Ectothiorhodospira halophila* grow well in the medium of Imhoff and Trueper (see Isolation of *Ectothiorhodospira halochloris*). In pure cultures, it is not necessary to add yeast extract. As sulfide feeding solution for these extreme halophiles, we recommend the following:

Distilled water	100 ml
$Na_2S.9H_2O$	5.0 g
$NaHCO_3$	10.0 g
NaCl	10.0 g

This solution is sterilized and fed to the cultures in amounts of 10 ml/liter.

Isolation and Growth of Phototrophic Bacteria

Irrespective of the source of the inoculum (*e.g.* sample from nature, enrichment culture, or suspension of a colony) the agar shake dilution method is the most convenient and reliable method to prepare pure cultures of phototrophic green and purple sulfur bacteria (Pfennig, 1965). For motile species that do not form colonies in agar media (*e.g.*, *Thiospirillum*) Giesberger's (1947) "Pasteur pipette" method

is recommended. For nonmotile species, the well-known dilution method in liquid media should be applied.

Agar Shake Dilution

Wash granular agar thoroughly several times with distilled water and then prepare 3 percent in distilled water (for marine samples, 2 *M* NaCl is added). Liquify the agar by autoclaving. While keeping it in a hot-water bath, dispense the agar in 3 ml portions into standard test tubes (16 or 18 mm X 200 mm). Plug the tubes with cotton and autoclave them.

Keep the molten agar in a water bath at 60° C. Keep a 50 or 100 ml screw-cap bottle, containing complete medium 1 (or medium 2 if this was used before), with a loosened screw cap in a water bath at 40° C. For one shake-dilution series, supply eight tubes with 6 ml of the prewarmed medium and keep them in the 40° C water bath. No shaking is required at this stage.

Inoculate one of the tubes with one to three drops from the suspension of the phototrophic bacteria. Mix the contents immediately by turning the tube once upside down and back (wetting the cotton plug does not disturb the further procedures). Then transfer 0.5-1.0 ml of the culture into a second tube that contains the agar medium. Mix immediately by turning as with the first tube. Continue this dilution series over eight steps. After transfer to the next tube, set each tube into a water bath with tap water to harden the agar. After hardening, seal the agar immediately with a sterile, liquified paraffin layer (one part paraffin dissolved in three parts of paraffin oil and autoclaved) of about 2-3 cm thickness. Keep the tubes in the dark for several hours, then reheat the paraffin layer of the tube to achieve a better seal. If slow-growing phototrophs are expected, replace the air above the paraffin layer by gassing with N_2 and close the tubes tightly with rubber stoppers. Incubate the agar shake-dilution cultures at 20-28° C (depending on the previous treatment of the inoculum) at a light intensity of 200-500 lux.

When the cultures have developed, isolate the individual colonies that show pigmentation from the highest dilution step. To do this, remove the paraffin layer by melting it. Then remove a single colony through the open end of the test tube by suction, using a fine Pasteur pipette attached to a rubber tube. The procedure is best carried out under a dissecting microscope.

Suspend the content of the colony in 0.5 ml of sterile medium in a test tube. Check the suspension microscopically for purity, and then repeat the whole dilution series in agar shakes. In order to obtain a pure culture, it may be necessary to repeat the whole process again. When pure cultures and colonies are achieved, inoculate individual colonies into liquid medium. It is advisable to start with small-sized bottles or screw-cap tubes (10 ml, 25 ml) and then to scale up to the regularly used sizes.

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CHAPTER III
ECOLOGY OF METHANOGENESIS:

DISTRIBUTION, PHYSIOLOGY, AND CARBON STABLE-ISOTOPE FRACTIONATION

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INTRODUCTION TO BACTERIAL METHANOGENESIS

In most anoxic environments the ultimate fate of organic material is as either a gaseous end product (methane or carbon dioxide) or as sequestered carbon. The production of methane is called methanogenesis and is carried out by a group of organisms called methanogens. The following experiments were pursued to elucidate some of the physiological and ecological parameters of these organisms. The ecological studies were conducted in an anoxic salt water sediment community commonly referred to as the Embarcadero Road Salt Marsh. The physiological studies encompassed the identification of isotope fractionation patterns in *Methanobacterium thermoautotrophicum* and the effects of interspecies hydrogen transfer and bromoethanesulfonic acid (BES) on the metabolism of *Methanococcus voltae* and *Methanosarcina barkeri*.

In many lakes and coastal regions, biologically produced methane is an important component of the complex microbial food-web. In anoxic environments, the methanogens utilize acetate, methanol, methylated amines and hydrogen, all of which are end products of fermenting bacteria. Our studies were designed to investigate these crucial questions toward an understanding of bacterial methanogenesis:

Where, at what rates, and from which chemical precursors is methane produced in a salt marsh environment?

How are carbon stable isotopes fractionated in a methanogenic metabolism and how can fractionation effects be used to decipher the complex processes of global anaerobic carbon cycling?

What are the dynamics of hydrogen transfer between methanogens and other bacterial species?

To define the chemical environment of the methane producing bacteria the concentrations of CH_4, CO_2, SO_4, and CH_3COOH were measured relative to depth. Sediment samples were enriched with one of these methanogenic substrates: CO_2-H_2, trimethylamine, methanol or acetate. Methanogen cell densities were determined using the most probable number technique as a function of depth. Net methane production, ^{14}C-labeled acetate, and methionine conversion to methane and carbon dioxide were determined.

The higher than predicted growth rates of hydrogen utilizing bacteria (sulfate reducers and methanogenic bacteria) under limiting growth conditions may have been an outcome of molecular hydrogen transfer. This phenomenon, called interspecies hydrogen transfer, was studied using two different approaches.

Combined Culture:

Does hydrogen production by phototrophs occur in a mixed culture in which the second organism removes hydrogen (seen as a hydrogen sink)? The experiment was performed by establishing culture conditions suitable for methanogenic bacteria as well as phototrophic bacteria and cyanobacteria. After cultural conditions are obtained, combinations of methanogens, and phototrophic bacteria analysed for CH_4 to substantiate the transfer of molecular hydrogen.

Separate Culture:

Does separate culture physically limit the bacterial interaction to atmospheric mixing? This experiment was designed to directly measure the effect of the hydrogen waste product of one species as a substrate for a second. The effect of hydrogen transfer via the gas phase on the growth of both organisms was studied.

The stable isotopic fractionation of carbon and nitrogen by *Methanobacterium thermoautotrophicum* was examined. Cultures of this organism were grown in a medium consisting of inorganic salts through which a H_2-CO_2 gas mixture was bubbled. The flow rate of the gas mixture was high enough to prevent "closed system" effects of isotope fractionation. The ability of these organisms to fractionate carbon and nitrogen could be determined by limiting them to the single sources: CO_2 and NH_4^+, of known isotopic compositions. Measurements of the resulting cell mass, media and gaseous products (CO_2 and CH_4) allowed the fractionation patterns to be determined and a total carbon budget for the system to be established.

FIELD STUDIES

(Marc Alperin, John Bullister and Katherine Kuivila)

Site Description

The anaerobic sediment in the salt marsh at Embarcadero Road in the Palo Alto Baylands, provided an excellent site in which to examine factors affecting biological methane production. The sampling site consisted of an area of open flooded soil, harboring cyanobacteria and phototrophs at the surface, surrounded by salt marsh vegetation. Cores were taken on July 8, 15, and 21, 1982 when water levels were about 3.3, 2.4, and 3.6 feet above sea level, respectively. The temperatures of the sediments ranged from 18° to 20° C with Eh ranging from -380 mv at 1.0 cm to -420 mv at 40 cm. The salinity of the overlying water was about 2.2 percent.

The Chemical Environment

Sediment Methane Concentrations

An obvious indication of methanogenic activity is, of course, sediment methane concentrations. There are relatively high concentrations of methane commonly encountered in anoxic sediments. Methane present in the sediment is relatively insoluble in warm water, and migrates easily by bubble formation. Unfortunately, elaborate *in situ* sampling devices are required in order to perform precise measurements. Without the devices, methane depth profiles may show considerable scatter and variability. Despite this problem, methane depth distributions can provide a framework on which more sensitive indicators of methanogenesis can be based.

Three methane depth distributions were obtained throughout the month of July (Tables III-I and Figs. III-1 to III-3.) The extreme variability among the three profiles prevented us from generalizing in regard to methanogenesis in this environment. The profile from July 8 showed a moderate methane concentration (0.5 *mM*) in the upper 10 cm, slowly increasing to about 0.8 *mM* at 40 cm. The profile of July 15 showed an unusually sharp maximum of 1.2 *mM* at 5 cm, and a sharp decrease to 0.1 *mM*. The profile from July 21 showed a relatively high concentration in the upper 10 cm (1.5 *mM*) increasing and oscillating below 15 cm.

FIG. III-1
CONCENTRATION OF METHANE IN CORE
7/08/82

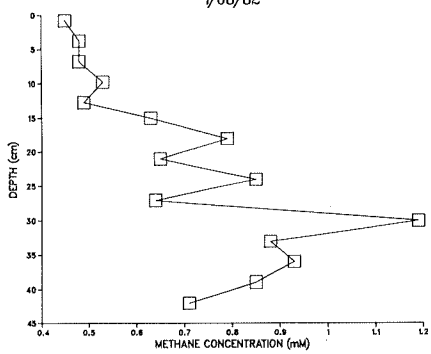


FIG. III-2
CONCENTRATION OF METHANE IN CORE
7/15/82

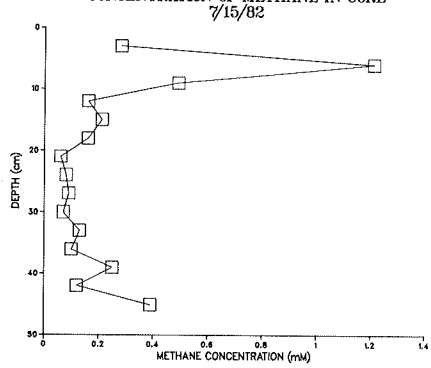


FIG. III-3
CONCENTRATION OF METHANE IN CORE
7/21/82

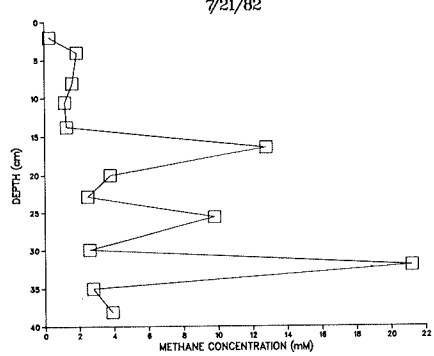


Table III-1

Methane Concentration in Pore Water

Core 7/8/82		Core 7/15/82		Core 7/21/82	
Depth (cm)	Methane Conc. (mM)	Depth (cm)	Methane Conc. (mM)	Depth (cm)	Methane Conc. (mM)
0- 1.5	0.45	2.2 - 3.7	0.28	1- 2.5	0.3
3- 4.5	0.48	5.2 - 6.7	1.21	4- 5.5	1.9
6- 7.5	0.48	8.2 - 9.7	0.49	7- 8.5	1.6
9-10.5	0.53	11.2 -12.7	0.16	10-11.5	1.2
12-13.5	0.49	14.2 -15.7	0.21	13-14.5	1.3
15-16.5	0.63	17.2 -18.7	0.16	16-17.5	12.0
18-19.5	0.79	20.2 -21.7	0.06	19-20.5	3.8
21-22.5	0.65	23.2 -24.7	0.08	22-23.5	2.5
24-25.5	0.85	26.2 -27.7	0.09	25-26.5	9.8
27-28.5	0.64	29.2 -30.7	0.07	28-29.5	2.6
30-31.5	1.19	32.2 -33.7	0.13	31-32.5	21.2
33-34.5	0.88	35.2 -36.7	0.10	34-35.5	2.8
36-37.5	0.93	38.2 -39.7	0.25	37-38.5	3.9
39-40.5	0.85	41.2 -42.7	0.12		
42-43.5	0.71	44.2 -45.7	0.39		

There can be several sources of this variability, one of which was undoubtedly due to the sampling technique. The methane analysis had a standard deviation of less than two percent, and therefore could not explain the scatter. When a core was inserted there was compression of the sediment and methane ebullition could be observed. The sharp oscillations seen in all three profiles may have been an artifact of bubble formation and migration caused by coring the sediment. On the other hand, the shift in the methane profile from day to day may have been related to tidal flooding and draining of the salt marsh.

Interstitial Water Sulfate Concentrations

In anoxic marine sediments, sulfate reducing bacteria are often dominant. These sulfate reducers may compete with the methanogens for the common substrates hydrogen and acetate. Interstitial water sulfate concentrations were determined in order to understand the extent of the sulfate reduction zone. The two sulfate depth profiles, Table III-II and Figs. III-4-5, agreed fairly well. In the upper 2 cm, sulfate concentrations were close to those found in the overlying water (14 mM). Both profiles showed a rapid sulfate decline with depths to about 5 cm, at which point the sulfate concentration which was 7-9 mM remained constant.

FIG. III-4
CONCENTRATION OF SULFATE IN CORE
7/08/82

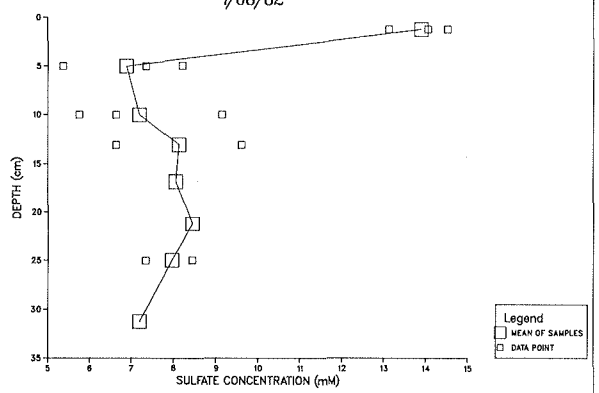


FIG. II-5
CONCENTRATION OF SULFATE IN CORE
7/21/82

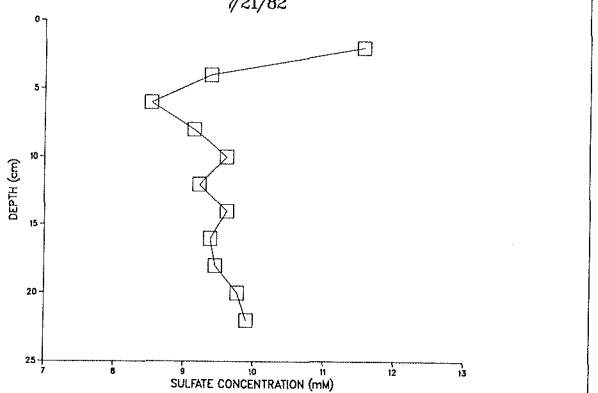


Table III-II
Sulfate Concentration in Pore Water

Core 7/8/82		Core 7/21/82	
Depth (cm)	Methane Conc. (mM)	Depth (cm)	Methane Conc. (mM)
0- 2	13.9	0- 2	11.5
4- 6	6.9	2- 4	9.1
8-10	7.1	4- 6	8.3
12-i4	8.1	6- 8	8.9
16-18	8.0	8-10	9.3
20-22	8.3	10-12	9.0
24-26	7.9	12-14	9.3
28-30	7.2	14-16	9.2
		16-18	9.3
		18-20	9.4
		20-22	9.6

Total Dissolved CO_2

Total CO_2 is a crude indicator of total heterotrophic activity. As organic carbon is decomposed by bacteria, CO_2 is produced. The total CO_2 depth profile is presented in Table III-III and Fig. III-6. The increase in total CO_2 with depth is a common feature of organic rich coastal sediments. The initial large concentration gradient in the upper 5 cm was maintained by relatively rapid rates of decomposition. Below 5 cm, the concentration gradient declined, suggesting a slower rate of net bacterial metabolism.

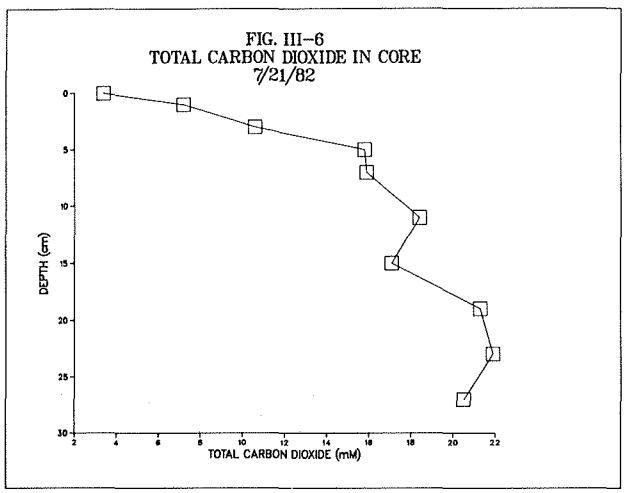


Table III-III

Concentration of CO_2 in Core Samples

Depth	CO_2, mM
overlying water	3.6/3.2/3.4
0- 2 cm	7.3/7.7/6.5
2- 4 cm	10.4/9.9
4- 6 cm	16.1/15.4/16.4/15.4
6- 8 cm	14.6/16.3/16.8/16.0
10-12 cm	19.6/18.7/19.0/16.1
14-16 cm	17.9/16.3
18-20 cm	19.1/19.3/23.7/22.9
22-24 cm	22.7/20.9/22.3/21.8
26-28 cm	19.8/21.2

Interstitial Water Acetate Concentrations

Acetate, present in fairly high concentrations relative to other fatty acids, is known to be a common substrate for both methane production, sulfate reduction, and heterotrophic assimilation. It is also known to have a very short residence time in sediment.

Previous studies of acetate concentrations and acetate turnover in marine sediments have suggested that a significant portion of the acetate pool is absorbed and not metabolically available. In an attempt to determine the active pool of acetate in the sediment samples, acetate kinase was employed.

Unfortunately, this technique was not sensitive enough, and the interstitial water from all depths examined had acetate concentrations below the detection limit (50 μM).

Methanogenesis

Most-Probable-Number of Methanogens

A most-probable-number (MPN) study was conducted on sediment samples from the salt marsh to determine indirectly the number of methanogens as a function of sediment depth (*Standard Methods for the Examination of Water and Wastewater*, thirteenth edition, 1971).

A core was sampled from the sediment surface (zero cm) to a depth of 28 cm in order to determine an MPN profile (7/8/82). A second sediment core sampled from zero cm to a depth of 12 cm was taken for a more detailed MPN study (7/15/82). The media used are given in the Appendix.

The largest MPN values occurred at depths between 2 and 8 cm (7/8/82). The Most Probable Numbers of methanogens determined for VMC-H_2-CO_2 and trimethylamine (VMC-TMA) media as a function of depth are shown in Table III-IV. On VMC-H_2-CO_2 media the Most Probable Number was 4.8 x 10^4, the methanogens seemed to be evenly distributed per 1 ml of wet sediment for all depths sampled. The MPN number for the VMC-TMA medium was largest for sediment core depths of 2 to 6 cm (4.8 x 10^4 cells per ml).

Table III-IV

Depth Distribution of Methanogens from Sediment Core

Depth (cm)	VMC-H_2-CO_2 Medium ($x10^3$ cells per ml)	VMC-TMA Medium ($x10^3$ cells per ml)
0- 2	48 (1)	4.6 (1)
2- 4	48 (1)	48 (2)
4- 6	48 (1)	48 (2)
6- 8	48 (1)	4.6 (1)
8-10	48 (1)	4.6 (1)
10-12	48 (1)	4.6 (1)

(1) = 95% Confidence Limit; 7,200 to 260,000.

(2) = 95% Confidence Limit; 800 to 24,000.

Methanogens capable of utilizing trimethylamine were maximal at sediment core depths of 2 to 6 cm. Statistically, there was a large range of error for the computation of these MPN's; (see Table III-IV for 95 percent confidence limits). This range could be narrowed significantly by an increase in the number samples from 3 to 5.

Enrichments for Methanogens

Methanogens have been shown to utilize a variety of substrates in the laboratory, including H_2-CO_2, acetate, methanol, mono-, di-, and trimethylamine. In the natural environment, the availability of these substrates and competition with other organisms determines their importance and the resulting rates of CH_4 production. Enrichment of sediment samples with the different substrates gives a first approximation of their utilization in the environment.

A sediment core was sampled at 2 cm intervals and the sediment put into N_2-CO_2 flushed serum vials. Boiled distilled water was added to make a slurry. Enrichments for methanogens were made by adding one of the substrates: H_2-CO_2, trimethylamine, methanol or acetate. The samples were incubated at 37° C. The headspace was sampled for CH_4 over time. The enrichment experiment was repeated (7/8/82 and 7/15/82).

The H_2-CO_2 enrichments showed a steady rate of CH_4 production until 24 hours had passed when the CH_4 increased rapidly (Table III-V and III-VI). Initial production rates were calculated from the slope of the curve of the CH_4 versus time. The sample from 6-8 cm had the highest initial CH_4 production rate, while the deeper samples showed a general decrease in CH_4 production rate relative to depth. The H_2-CO_2 enrichments from the second sampling had much lower initial CH_4 production rates with the maximum rates at 2-4 cm. Both methanol and trimethylamine containing media showed a maximum production rate at the 0-2 cm interval and a decrease relative to depth. The second set of samples (7/15/82) showed low production rates throughout for methanol and trimethylamine, with a small maximum at 4-6 cm, and a definite enrichment beginning after 20 hours. The acetate enrichments had only very low production rates at all depths for both sets of samples and no enrichment of acetate-utilizing methanogens.

Table III-V Initial CH_4 production rates ($mM\ CH_4$/hr) from Core I.

Depth	H_2/CO_2	Methanol	Trimethylamine	Acetate
2- 4	0.031 (4)*	0.025 (3)	0.029 (3)	0.011 (4)
6- 8	0.637 (6)	0.017 (5)	0.005 (3)	0.018 (6)
10-12	0.035 (4)	0.013 (4)	0.006 (3)	0.002 (3)
14-16	0.013 (3)	0.015 (4)	0.008 (3)	0.010 (4)
18-20	0.002 (4)	0.013 (2)	0.006 (3)	0.003 (6)
22-24	0.009 (3)	0.005 (6)	0.009 (6)	0.003 (4)
26-28	0.044 (4)	0.007 (3)	0.010 (6)	0.006 (6)
30-32	0.020 (4)	0.005 (4)	0.007 (5)	0.006 (5)
34-36	0.015 (4)	0.005 (5)	0.004 (5)	0.003 (5)
38-40	0.026 (5)	0.007 (3)	0.005 (5)	0.004 (5)

Slope from plot (least squares fit) of CH_4 vs. time, just using initial points (until no longer linear).

*Figures in parentheses indicate number of points out of 7 used in the linear portion.

Table III-VI Initial CH_4 production rates ($mM\ CH_4$/hr) from Core II.

Depth	H_2/CO_2	Methanol	Trimethylamine	Acetate
0- 2	0.016 (4)*	0.008 (4)	0.008 (4)	0.005 (3)
2- 4	0.072 (5)	0.005 (5)	0.005 (3)	not detectable
4- 6	0.004 (4)	0.011 (4)	0.011 (4)	0.004 (7)
6- 8	0.004 (4)	0.005 (5)	0.002 (3)	0.002 (7)
8-10	0.005 (3)	0.005 (5)	0.004 (5)	0.002 (6)
10-12	0.002 (4)	0.002 (5)	0.003 (4)	not detectable

*Figures in parentheses indicate number of points out of 7 used in the linear portion.

The enrichment experiments suggest the relative importance of the four substrates, CO_2-H_2, methanol, trimethylamine and acetate, but cannot predict *in situ* rates of CH_4 production. H_2-CO_2 seemed to be the major source of methane. The first set of samples with an initial CH_4 production rate 20 times higher than any of the other rates, seemed questionable. But scatter in the data correlated with depth suggested errors in sampling. The initial production rates from methanol and trimethylamine indicated some utilization at 2-4 cm with decreasing importance relative to depth. The low

production rates from acetate suggested that it was the least important substrate in this environment. The second set of enrichments showed much less scatter and was better correlated with depth. H_2-CO_2 appears to be the preferred substrate. Rates of methane production were lower than the rates found in the first set of enrichments. The maximum production rate was at 2-4 cm instead of 6-8 cm, again decreasing relative to depth. Methanol, trimethylamine and acetate all showed decreased rates from the first enrichments and a slight maximum at 4-6 cm for all three substrates. Both experiments inferred that the preferred methanogenic precursors were H_2-CO_2, methanol, trimethylamine, and then acetate.

Sediment Methane Production Rate

To examine the rate at which methane is produced in the salt marsh, slurries from various depths in the sediment were incubated in serum vials for 6.5 days. Any methane that accumulated in the headspace during this time would represent the net result of several complementary or opposing processes, *i.e.*, bicarbonate reduction, acetate fermentation, and/or methane oxidation.

However, methane accumulated in the headspace of the serum vials at rates slower than 50 n*M*/g per sediment per day. The uncertainty in measuring rates this slow is such that the numbers are not statistically significant. We can only say that methane production rates in the upper 30 cm of the sediment were slower than 100 μM per day.

To investigate the rate at which methane was produced from bicarbonate a more sensitive radiocarbon tracer experiment was undertaken. ^{14}C-O_2 was injected into a sediment slurry as sodium bicarbonate. Samples were incubated for 6 days at room temperature and the $^{14}CH_4$ was collected. The rates of methane production were very low for the sediment column (Table III-VII). At 2-6 cm, a rate of 0.15 μM per day was estimated. Deeper in the sediments, no $^{14}CH_4$ was produced. Methane production from 2-^{14}C-acetate and methyl-labeled methionine was also examined (Tables III-VIII and III-IX). These experiments only provided information about the utilization of these substrates by methanogens in the salt marsh sediment because no pool site information was available either for acetate or methionine. Both acetate and methionine were converted to methane in the sediment. The proportion of acetate metabolized to methane relative to its oxidation to CO_2 increased with depth, although it never exceeded 5 percent. On the other hand, the ratio of labeled methane to labeled CO_2 for the methionine experiment reached 20 percent.

Table III-VII
Methane Production Rates from ^{14}C-carbon dioxide

Depth (cm)	cpm CH_4	cpm CO_2	ΣCO_2 mM	Rate ($\mu M\ day^{-1}$)
2- 6	27	299,000	13.0	0.15
6-10	0	281,000	16.0	< 0.15
10-14	0	300,000	18.0	< 0.15
14-18	0	307,000	18.0	< 0.15
20-24	0	275,000	21.6	< 0.15
26-30	0	268,000	20.5	< 0.15

All values reported have been corrected for the control.

383,000 cpm of $H^{14}CO_3^-$ were added to each sample.

Table III-VIII
Methane Production Rates from $^{13}CH_3$-acetic acid

Depth (cm)	cpm CH_4	cpm CO_2	(cpm CH_4/cpm CO_2)·1000
2- 6	56	122,000	0.5
8-12	2,700	114,000	24.0
14-18	1,000	121,000	8.4
20-24	3,400	74,000	45.5
28-32	*	32,000	----

*lost CH_4 during stripping

195,000 cpm of $^{14}CH_3COOH$ were added to each sample.

All values reported above have been corrected for the control.

Table III-IX
Methane Production Rates from $^{14}CH_3$-methionine

Depth (cm)	cpm CH_4	cpm CO_2	(cpm CH_4/cpm CO_2)·100
2- 6	988	9,000	109
8-12	390	13,000	29
14-18	245	5,000	48
20-24	173	775	223
28-32	0	382	0

36,300 cpm of ^{14}C-methionine were added to each sample.

All values reported above have been corrected for the control.

GROWTH AND METHANE PRODUCTION BY *METHANOSARCINA BARKERI*

(Katherine Kuivila)

Coenzyme M, recently discovered and thought to be unique to methanogens, is involved in the terminal step of methane production. Bromoethanesulfonate (BES), an analogue of this coenzyme, has been shown to be a potent inhibitor of the methyl-coenzyme M reductase in *Methanobacterium thermoautotrophicum* (Gusalus *et al.*, 1978). The addition of concentrations of 7.9×10^{-4} *M* BES to the cultures of this bacterium resulted in 50 percent inhibition of methane production. This study examined the inhibition by BES of CH_4 production by another methanogen, *Methanosarcina barkeri*, when grown on H_2-CO_2 or methanol.

The rate of production of methane by *M. barkeri* growing with CO_2-H_2 or methanol as a carbon source was determined (Table III-X and Fig. III-7). After a lag of approximately 20 hours, the H_2-CO_2 culture began growing at a steady rate. Varying concentrations of BES were added after 89 hours (0 to 1.0×10^{-4} *M*). The production of methane was not affected by the addition of the inhibitor. However, both the absence of optical density change, and the relatively slow rates of CH_4 production suggested that the cultures were still in the lag phase of growth even after 89 hours, which could account for the apparent lack of the inhibition of CH_4 production. The sudden drop in CH_4 concentrations observed in two of the cultures (without BES and the one containing 1×10^{-7} *M* BES) was probably due to loss of methane during addition of BES.

With methanol (0.1 *M*) as the carbon source, no growth occurred in 62 hours, as determined by a lack of methane production, and by lack of an increase in optical density. The experiments should be repeated after the cultures have reached log phase.

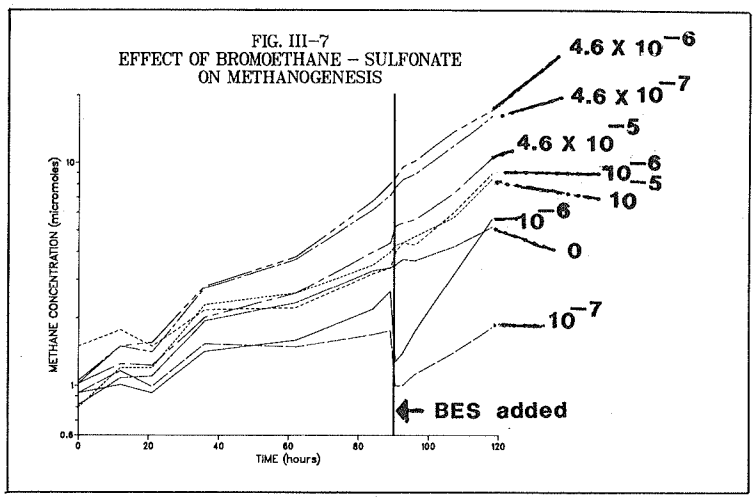


Table III-X Methane Production in the Presence of BES

#	Time ------ (hours) 0	12	21	36	62	84	88.5	90.5	92.5	96	107	118	BES
1	0.932	1.02	0.925	1.42	1.59	2.36	2.53	1.27	1.38	1.74	3.18	5.57	0
2	0.928	1.17	0.998	1.55	1.48	1.70	1.76	0.937	0.916	1.12	1.42	1.82	1×10^{-7} M
3	1.05	1.49	1.41	2.77	3.69	6.21	7.18	7.82	8.37	8.88	11.9	16.2	4.6×10^{-7} M
4	1.50	1.79	1.49	2.17	2.23	3.22	3.49	3.95	4.40	4.27	6.08	9.00	1×10^{-6} M
5	1.03	1.50	1.55	2.81	3.79	6.82	8.13	8.74	9.74	10.2	13.8	18.8	4.6×10^{-6} M
6	1.02	1.26	1.22	2.04	2.61	3.49	3.98	4.29	4.37	4.68	5.74	8.41	1×10^{-5} M
7	0.802	1.20	1.22	2.29	2.58	4.02	4.41	5.32	5.41	5.47	7.33	10.7	4.6×10^{-5} M
8	0.801	1.07	1.11	1.98	2.32	3.29	3.38	3.51	3.71	3.67	4.11	5.08	1×10^{-4} M

CH_4 = µmoles

BES was added at 89 hours.

Methanosarcina barkeri

GROWTH AND ISOTOPE FRACTIONATION BY *METHANOBACTERIUM THERMOAUTOTROPHICUM*

(John Bullister and Rodger Harvey)

Stable carbon isotopes can be used to study metabolic processes of and carbon transportation in organisms. In anaerobic environments, methane production plays a significant role in the distribution and cycling of carbon containing

compounds. The isotopic composition of methane produced in anaerobic zones, extremely enriched in light carbon, can significantly alter the isotopic composition of the carbon available to the aerobic milieu. Laboratory cultures of methanogenic bacteria were grown in order to gain insight into the potential that these organisms have to fractionate carbon during the production of methane and cell biomass. *Methanobacterium thermoautotrophicum*, of which a pure culture was brought to the course by L. Baresi, was used. This methanogenic bacterium required only a simple medium of inorganic salts and H_2-CO_2; the sole carbon source of this obligate chemolithoautotroph was CO_2. The organism had a generation time of about 3 hours at 65° C, allowing high cell densities and rapid methane production to be achieved within a few days after inoculation.

By providing a single source of carbon (H_2-CO_2 from a gas cylinder), and equilibrating the bicarbonate/carbonate in the medium with this gas before introducing the organism, carbon of known isotopic compositions was initially supplied to the cells. The subsequent bubbling rate of CO_2 through the medium was rapid enough to minimize depletion of light carbon in the CO_2 leaving the system, and to maintain a *delta* ^{13}C value close to that present initially. Because the *delta* ^{13}C introduced was maintained constantly, subsequent patterns of carbon fractionation into CH_4 and cell material could be resolved. This "open" system contrasts to a "closed" system, where the *delta* ^{13}C of a limited substrate constantly changes during the course of an experiment.

The *delta* ^{13}C of the CO_2 entering and leaving the culture, CH_4 produced by the culture, carbon in the cell material, and crude lipid fraction at the time of harvest were measured. The results are summarized in Table III-XI. The flow rate of the H_2-CO_2 gas through the system, and the concentration of methane in the effluent gas were determined for each run. The *delta* ^{13}C of the incoming and outgoing CO_2 differed only slightly (approximately 1-3 percent) (Table III-XI). In an "open" system, with an essentially infinite flow rate, no change in *delta* ^{13}C would be expected between the incoming and outgoing CO_2. The small difference actually observed can be attributed to the selective removal of (isotopically) light CO_2 by the organisms as they produced CH_4 and cell material.

Table III-XI Stable Carbon Isotope Fractionation by *Methanobacterium thermoautotrophicum*

	Culture I	Culture II	Culture III
Temp. (°C)	56	56	66
Flow (ml/min)	140	192	92
CO_2 (in)	- 38.2 ‰	lost	- 39.25 ± 0.5 ‰
CO_2 (medium)	- 29.8 ± 2.6 ‰ $\bar{x} = 3$	- 30.7 ± 2.0 ‰ $\bar{x} = 3$	- 28.5 ± 1.4 ‰ $\bar{x} = 3$
CO_2 (out)	- 37.3 ± 0.6 ‰ $\bar{x} = 2$	- 38.0 ± 0.1 ‰ $\bar{x} = 3$	- 36.7 ± 0.1 ‰ $\bar{x} = 2$
CH_4 (out)	- 73.5 ± 2.4 ‰ $\bar{x} = 3$	- 65.14[c] $\bar{x} = 1$	- 77.4 ± 0.7 $\bar{x} = 3$
CH_4 concentration $CO_2 \dashrightarrow CH_4$	0.97% 1.043	0.57% ---	1.18% 1.049
CO_2 (ℓ)[a]	- 33.4 ‰	- 34.3 ‰	- 32.0 ‰
intact cells	- 62.9 ± 0.3 ‰ $\bar{x} = 3$	- 62.97 ± 0.5 ‰ $\bar{x} = 3$	- 62.35 ‰ $\bar{x} = 1$
total lipids	ND[b]	- 65.8 ± 0.2 ‰ $\bar{x} = 3$	- 65.03 ± 0.6 ‰ $\bar{x} = 3$

[a] assume $HCO_3^-/CO_2 = 4:1$; assume $pK_a = 6.4$; $\delta CHCO_3 - \delta CCO_2 = 4.58$ ‰

[b] not determined

[c] methane sample analyzed for isotopic composition was only slightly larger than system blank

A more significant difference in *delta* ^{13}C was seen between the incoming CO_2 and the total CO_2 dissolved in the medium at the time of harvest. As CO_2 gas dissolved in water, the following reaction occured:

$$CO_2\ (g) \longrightarrow CO_2\ (l)$$
$$CO_2\ (l) + H_2O \longrightarrow HCO_3^- + H^+$$
$$HCO_3^- \longrightarrow CO_3^{2-} + H^+$$

At equilibrium, the ratio of dissolved HCO_3^- to CO_2 (l) was 4:1 at pH 7.

In addition to chemical equilibrium, an isotopic equilibrium is established as CO_2 (l) $\longrightarrow HCO_3^-$ which produced the following isotopic fractionation.

T = 56.3°C *delta* $^{13}C = 4.56°/_{oo}$
T = 65°C *delta* $^{13}C = 4.36°/_{oo}$

The actual differences in isotopic composition observed between the incoming gas and total CO_2 in the medium was greater

than that expected from single chemical and isotopic equilibrium. These differences were due to depletion of light carbon in the medium by the growing methanogens. The result was a shift in the isotopic composition of all the components in the media (CO_2 (1), HCO_3^- and $CO_3^=$) towards heavier values. From the *delta* ^{13}C value of the total CO_2 of this medium, the *delta* $^{13}CO_2$ (1) *etc.* in the medium can be calculated.

For Culture 1, *delta* ^{13}C of the medium = -29.8 $^o/_{oo}$.
Therefore, since *delta* $^{13}CO_2$ (1) = *delta* $H^{13}CO_3^-$ -4.56 $^o/_{oo}$ and 1.
(*delta* $^{13}CO_2$) + 4(*delta* $H^{13}CO_3^-$) = 5(-29.8) $^o/_{oo}$
delta $^{13}CO_2$ (1) = -33.4 $^o/_{oo}$

Calculated values for *delta*-$^{13}CO_2$ for the other cultures are given in Table III-XI.

The large enrichment in isotopically light carbon present in this organism indicated uptake of CO_2 (1) rather the than other forms of carbon. Therefore, the fractionation factors for other components were calculated relative to this value and are also shown in Table III-XI.

As can be seen from Table III-XI, cellular isotopic composition did not vary significantly between the cultures examined at the two growth temperatures. The slightly heavier value of intact cells grown at 65 degrees could not be considered significant due to loss of replicate samples during processing. These results contrasted with the results of other investigators (Simon and Plum, 1966; Belyaev, et al., 1983), who found significant differences in cellular isotopic composition of cells grown at various temperatures. Cultural techniques, however, as well as phase of growth at which cells were harvested (stationary vs. log) may have accounted for the differing conclusions.

As with the cellular carbon isotopic composition, total lipid values for 56 and 65° C showed a consistent carbon composition averaging only 2.7 to 2.8 $^o/_{oo}$ lighter than intact cells at such temperatures. Although other routes for intracellular fractionation of elements were not examined (proteins, amino acids, excreted metabolites), such small differences in lipid values versus the total carbon pool may have indicated fractionation at the initial assimilation stage. The large fractionation of CO_2 as it entered the cell (*delta* cell - *delta* CO_2 = -29.5 $^o/_{oo}$) may have occurred at the initial fixation step of CO_2 in this autotrophic organism, and not after incorporation.

The methane produced by an actively growing culture of *M.*

thermoautotrophicum contained the greatest enrichment in ^{13}C of the microbial components examined. In growing cells, greater than 90 percent of the CO_2 consumed reappeared as methane, while less than 10 percent remained as cellular carbon. Moreover, the isotopic fractionation of methane (Table III-XI) was significantly greater than the fractionation seen for intact cells. This indicated a larger fractionation of initially fixed CO_2 than was evident from comparison of intact cells and CO_2 in the medium.

By measuring the amount and isotopic composition of the carbon species entering and leaving the system, as well as any changes inside the system due to cell growth, a carbon budget could be constructed. In culture 1, the flow of carbon dioxide through the system was approximately 21,000 mgC/day (at STP) and methane production rate at time of harvest was approximately 770 mgC/day. New production of cell carbon was 770 mgC/day. From this data, the dominant components of this system were CO_2 in, CO_2 out, and CH_4 out.

Within the precision of the measurements, a reasonably well balanced carbon budget could therefore be constructed considering such gas equilibria.

For the first experiment:	concentration of gas present
delta CO_2 (in) = -38.2 $^o/_{oo}$	20.00 percent CO_2
delta CO_2 (out) = -37.3 $^o/_{oo}$	19.03 percent CO_2
delta CH_4 = -73.5 $^o/_{oo}$	0.97 percent CH_4

delta CO_2 (in) (CO_2 (in)) = *delta* CO_2 (out) (CO_2 (out)) + *delta* CH_4 (out) (CH_4 (out))

The fractionation of CO_2 into CH_4 and cell material reported here for *M. thermoautotrophicum* is reasonably close to reported values from *in situ* anaerobic sediments. Thus, light methane may be attributed to the metabolism of the methane producing organisms.

INTERSPECIES HYDROGEN TRANSFER

(Dieter Giani)

Phototrophic bacteria, including cyanobacteria, produce H_2 under special conditions. Phototrophic bacteria except for most cyanobacteria, withstand strongly reduced environmental conditions compatible with methanogenesis. Interspecies H_2 transfer between phototrophic and methanogenic bacteria has been hypothesized to occur. Indeed, phototrophic bacteria may even use CH_4 for reducing power. H_2 production by

phototrophic bacteria is especially prevalent during N_2 fixation. Excess H_2 may be produced if it is consumed by the methanogenic or sulfate reducing bacteria maintaining the partial pressure of H_2 at low levels.

Three approaches were made to search for interspecies H_2 transfer:

Cyanobacteria that consume either methane or H_2S were sought in enrichment experiments.

Pure cultures of phototrophic bacteria and methanogenic bacteria were physically combined.

Pure cultures of cyanobacteria and methanogenic bacteria, were connected only by the gas phase.

Enrichment Experiments

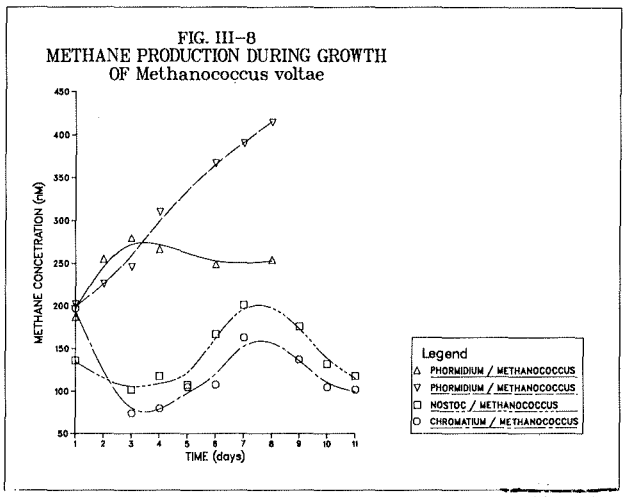
Mineral media differing in N source and nutrient addition were used to enrich methane or sulfide consuming cyanobacteria. Samples from Alum Rock Park and the salt marsh (0.5 ml) were inoculated in BG 11 (bluegreen), BG 11 (red), M, M/M (1/2 of trace minerals normally found in M) and BG/M (1/2 BG + 1/2 M), but neither visible growth nor methane production occurred.

Because both the salt marsh environment and the Alum Rock Park water contained methane, cyanobacterial enrichments of both sites were started, and incubated in the same media as above with 0.5 V/V CH_4 present. Methane ebullition through mats of phototrophic organisms was observed, at the salt marsh as well as at the Alum Rock Park site, where methane concentrations of 18 μM in the pristine spring water were found. While the salt marsh cyanobacterium (*Oscillatoria princeps*) lysed rapidly (1 day), the Alum Rock Park species withstood the conditions for at least 3 weeks, even though neither growth nor methanogenesis was observed.

Combined Culture Experiments

Combined cultures of *Methanococcus voltae* with *Chromatium vinosum*, *Nostoc muscorum* or *Phormidium luridium* were inoculated into modified VMC media. Except for the *P. luridium/M. voltae* combination, consistent production of methane was not observed even though growth of *C. vinosum* was observed (maximal cell density occurred within about 3 days independent of medium modifications) and H_2S or acetate was the suspected electron donor. Typical curves of methane content

of the bottles are shown in Figure III-8.



In the case of the cyanobacteria, probably none of the reduced compounds available in the medium could be used as electron donors because the water splitting system was blocked by the reduced conditions. Some H_2 might have come out of reduced carbon. Possibly photoheterotrophic cultivation could enable them to grow under these conditions.

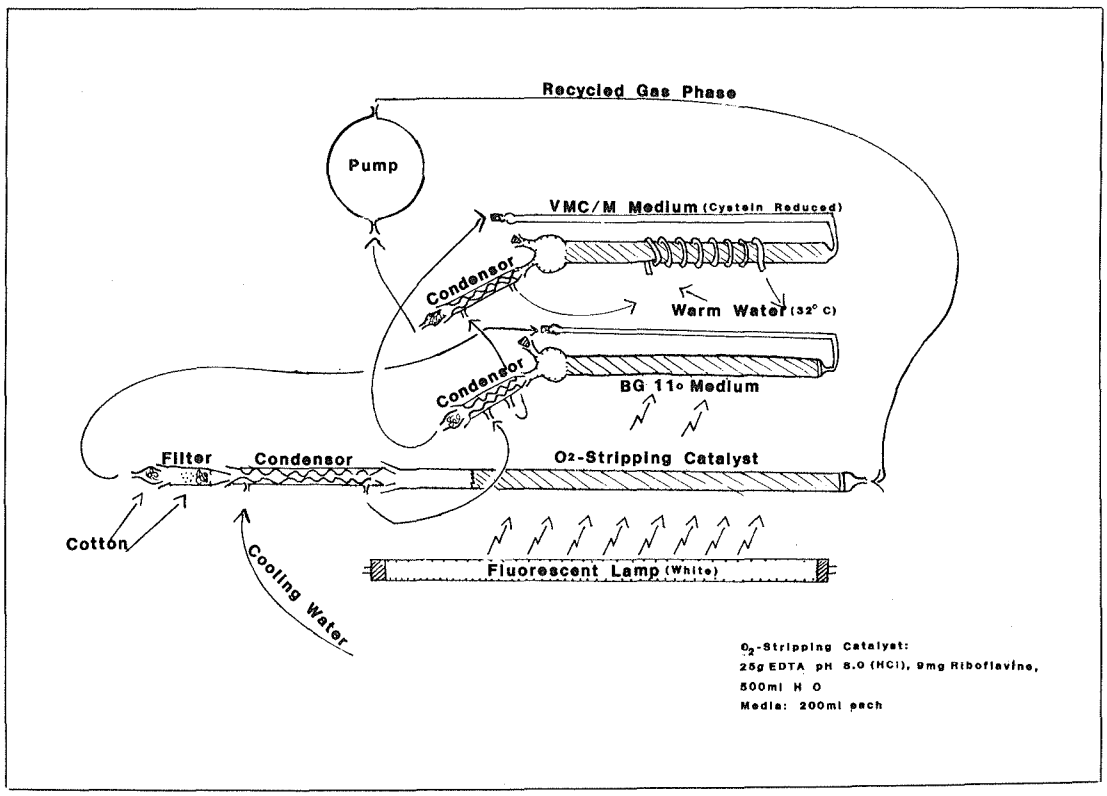
In the case of *Chromatium*, the methanogens were unable to grow fast enough to alter the metabolism of the phototroph. In our attempt to impede the growth of the phototroph, cultures were incubated at 37° C in a reduced light gradient, but *C. vinosum* did not grow. A suggested alternative to limiting the phototroph would be increasing the initial methanogenic population.

Separated Culture

The cyanobacterium *Plectonema boryanum* when grown under N_2 fixing conditions in a system as shown in Fig III-9, produced measureable amounts of H_2 in the gas phase. Therefore, an experiment was started in which a cyanobacterial culture was connected to a methanogenic culture through the gas phase (see Appendix III.) Unfortunately, *P. boryanum* was not available, and its substitute *Nostoc muscorum* did not grow under these conditions even after 9 days of adaption time. But the system itself worked well. Once reduced, the methanogenic medium did not become reoxidized as indicated by resazurin. Although it might have been possible to investigate the influence of H_2 uptake of methanogens and sulfate reducers on the H_2 production of cyanobacteria or other phototrophs using this equipment, the failure of the

cyanobacteria to grow and time limitation precluded any conclusion from this work.

Fig. III-9 **Apparatus for growth of *Plectonema boryonum* under nitrogen-fixing conditions.**



DISCUSSION

The salt marsh environment on Embarcadero Road offered an excellent opportunity for investigating ecological aspects of methanogenesis. The salt marsh sediment was an anoxic saline environment with visible ebullition of gases. Methane concentrations ranged from 0.1 to 20 m*M* and compared favorably with concentrations for a similar site in the San Francisco bay area reported by Dr. Oremland (1980), of 0.4 m*M*. The general distribution of methane with relation to the depth of the salt marsh was similar to other estuarine sediments analyzed by Martens and Berner, (1974) (Long Island Sound) and Whelan, (1974) (Louisiana salt marsh). In all studies the maximum concentration of methane was observed in the upper meter of sediment. But the specific distribution of methane differed in the three cores analyzed. We feel that the changes in the distribution reported here are valid. In the core sample taken July 15 the highest most probable number of methanogens occurred at the same depth as the maximum concentration of methane. (The

change in distribution of methane at different times may have been due to tidal changes.)

Preferred substrates for methanogenesis were H_2-CO_2, methanol, trimethylamine, and acetate (in order of preference). For the July 15 core the enrichments on H_2-CO_2 showed a maximum between 2-4 cm which is similar to that observed for dissolved methane and the most probable number of methanogens. It was not clear why the enrichment maximum in the core sample taken on July 8 was 6-8 cm above the 30 cm maximum for dissolved methane.

The rate of methane production from sediment cores using isotopes showed a maximum rate of methane production from CO_2 of 0.15 *uM* per day. Other investigators have found rates ranging from 0.2 to 1 μM per day (Cappenberg, 1974, and Barber, 1974). Both direct and indirect methanogenesis from acetate and methionine were observed but the rate of production could not be estimated due to the inability to accurately measure the pool sizes for these substrates.

The carbon isotope fractionation work on *M. thermoautotrophicum* supported and extended the work of Fuchs *et al.*, (1979). Little or no difference in the incoming *delta* ^{13}C was found. The lack of difference suggested that all *delta* ^{13}C work was done on an open system. The change of 31 $^{o}/_{oo}$ in *delta* ^{13}C (methane) {= *delta* ^{13}C(methane) - *delta* ^{13}C(carbon dioxide)} reported by Fuchs *et al.*, (1979) was similar to values reported here of 31 $^{o}/_{oo}$ and -35 $^{o}/_{oo}$. Such a large isotope fractionation suggests the active form of carbon for methanogenesis was CO_2 rather than bicarbonate. If bicarbonate had been used a smaller isotope effect would have been expected. (The smaller isotope effect is attributable to the higher mass and lower kinetic effects of bicarbonate. For example, CO_2 fixation reactions had *delta* ^{13}C(cell) greater than 20 $^{o}/_{oo}$ whereas for bicarbonate the values were less than 20 $^{o}/_{oo}$.)

Cell carbon fractionation patterns in *M. thermoautotrophicum* were also studied. Fractionation resided in the first step of CO_2 asimilation, as in methanogenesis. Analysis of the lipid fraction, a major component of cellular carbon, was only 2.7 to 2.8 $^{o}/_{oo}$ lighter than intact cells.

Isotope fractionation was only one of three physiological studies undertaken. The remaining two studies were

bromoethanesulfonic acid (BES) inhibition and interspecies hydrogen transfer. The BES inhibition experiments were developed in order to see if methanogenesis would be inhibited by a coenzyme M analogue. The methanogen best suited for this study grew poorly; results are equivocal. In the interspecies hydrogen transfer experiments the difficult problem of growing phototrophic bacteria with methanogenic bacteria was undertaken. Culture experiments, using pure culture and combined cultures of cyanobacteria and methanogenic bacteria, proved technically feasible. Encouraging results were obtained on mixed cultures of *Phoridum* and *M. voltae*. Successful application of mixed culture techniques could be used on organisms more acclimated to the technical restraints of the equipment.

SUMMARY

(Dr. Larry Baresi)

In conclusion both ecological and physiological studies of the process of methanogenesis and methanogenic bacteria were undertaken. Methane was found to be produced in the salt marsh environment with H_2-CO_2 being the preferred substrate. The distribution of dissolved methane was found to change at different times. One reason for this behavior may have been the constant change in the tide. Further work is necessary in order to determine the reason for this behavior. The rate of methanogenesis was well within the range of other observations at about 0.15 μM per day. Isotope fractionation studies suggested that carbon dioxide rather than bicarbonate was the active species in methanogenesis and in the synthesis of cell carbon. Interspecies hydrogen transfer experiments proved technically feasible. But, along with the BES experiments, the interspecies hydrogen transfer experiments need to be extended in order to meet with success.

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METHANOGENIC BACTERIA MEDIA AND TECHNIQUES

(Larry Baresi modified from R. S. Wolfe)

In 1950 R. E. Hungate devised a technique for culturing strict anaerobes which has since commonly been referred to as the Hungate Technique. Prior to 1950, no reliable method existed for the isolation and maintenance of pure cultures of strictly anaerobic bacteria found in black muds, in the rumen and intestinal tracts of animals, in sewage digesters, and in some wounds. The Hungate Technique depends on the elimination of oxygen from all cultural material. Gases are rigorously freed of oxygen and the media is pre-reduced in sealed tubes with rubber stoppers. Anaerobic procedures will be described here.

Methanogens are a morphologically diverse group of organisms with the common property of producing methane as a metabolic end product. Most cultures of methanogenic bacteria are able to grow by oxidizing H_2 using CO_2 as the terminal electron acceptor (Reaction 1, below). Some methanogens are also capable of oxidizing formate (Reaction 2). One group, the genus *Methanosarcina*, is capable of metabolizing methanol (Reaction 3), acetate (Reaction 4), and monomethylamine, dimethylamine, and trimethylamine. The names and selected characteristics of some methanogenic bacteria appear in Tables III-XII and III-XIII.

Typical Reactions of Methanogens:

1. $4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$
2. $4HCOOH \longrightarrow 4H_2 + 4CO_2 \longrightarrow CH_4 + 3CO_2 + 2H_2O$
3. $4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$
4. $CH_3COOH \longrightarrow CH_4 + CO_2$

Table III-XII Selected characteristics of pure culture methanogenic bacteria

Habitats	*Methanobacterium ruminantium*	*Methanomicrobium mobile*	*Methanobacterium formicicum*	*Methanobacterium brantii*	*Methanobrevibacter arboriphilus* strain	*Methanobrevibacter arboriphilus* AZ	*Methanobacterium thermoautotrophicum*	*Methanosarcina barkeri*	*Methanococcii vanniellii*	*Methanospirillum hungati*
Rumen	+	+	+							
Sewage sludge	+		+			+	+	+		+ a
Sediments			+	+	+ b			+	+	
Hot Springs							+			
Morphology										
Coccus										
Rod	short	short	long	curved	+	+	long		+	
Spirillum								sarcina		+
Size (μm)	0.7 x 1.8	0.7 x 2	0.4 x 15	0.4 x 15	0.6 x 8.7		0.4 x 7	2-10	0.5-4	0.5 x 7.4
Substrates										
H_2/CO_2	+	+	+	+	+	+	+	+	+	+
Formate	+	+	+						+	+
Methanol								+		
Acetate								+		
Gram Stain	+	-	variable	+	+	+	+	+		-
Motile	No	Yes	No	No	No	No	No	No	Yes	Yes
Growth Factors	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
Temperatures	37-40	40	37-45		30-37	33-40	65-70	35-40	37-42	30-42
pH optimum	6.5-7.7	6.1-6.9	6.6-7.8		7.5-8.0	7.0	7.2-7.6	6.5-7.0	8.0-8.5	6.8-7.5

a - also isolated from pear waste digester.

b - also isolated from tree interiors.

Table III-XIII **Determinative key to species of the methanogenic bacteria based on simple phenotypic characters.**

I. Gram-positive to gram-variable rods or lancet-shaped cocci often forming chains and filaments.
Order I. *Methanobacteriales*
Family I. *Methanobacteriaceae*
A. Slender, straight to irregularly crooked long rods often occurring in filaments.
Genus I. *Methanobacterium*
1. Mesophilic.
a. Methane produced from formate.
Methanobacterium formicicum
b. Methane not produced from formate.
Methanobacterium bryantii
2. Thermophilic
Methanobacterium thermoautotrophicum
B. Short rods or lancet-shaped cocci which often occur in pairs or chains.
Genus II. *Methanobrevibacter*
1. Cells form short, nonmotile rods which do not utilize formate.
Methanobrevibacter arboriphilus
2. Chain-forming, lancet-shaped cocci that produce methane from formate and require acetate as a carbon source.
a. Growth requirement for 2-mercaptoethanesulfonic acid and D-α-methyl butyrate.
Methanobrevibacter ruminantium
b. Do not have an obligate growth requirement for 2-mercaptoethanesulfonic acid or D-α-methylbutyrate.
Methanobrevibacter smithii
II. Gram-negative cells or gram-positive cocci occurring in packets.
A. Gram-negative, regular to slightly irregular cocci often forming pairs.
Order II. *Methanococcales*
Family I. *Methanococcaceae*
Genus I. *Methanococcus*
1. Cells inhibited by addition of 5% NaCl to medium.
Methanococcus vannielii
2. Cells not inhibited by addition of 5% NaCl to medium.
Methanococcus voltae
B. Gram-negative rods or highly irregular cocci occurring singly.
Order III. *Methanomicrobiales*
Family I. *Methanomicrobiaceae*
1. Straight to slightly curved, motile, short rods.
Genus I. *Methanomicrobium*
Methanomicrobium mobile
2. Irregular coccoid cells.
Genus II. *Methanogenium*
a. Cells require acetate.
Methanogenium cariaci
b. Cells do not require acetate.
Methanogenium marisnigri
3. Regularly curved, slender, motile rods, often forming continuous spiral filaments.
Genus III. *Methanospirillum*
Methanospirillum hungatei
C. Gram-positive coccoid cells which usually occur in packets and ferment methanol, methylamine, and acetate.
Family II. *Methanosarcinaceae*
Genus I. *Methanosarcina*
Methanosarcina barkeri

See Bergey's Manual (Buchanan and Gibbons, 1974)

Preparation of Prereduced Medium for Methanogens

Materials:

Hungate gassing station
80:20 N_2:CO_2 gas mixture
tubes or bottles
pipettes
stoppers
round bottom flask
medium
Bacto-agar

Step 1

Add 200 ml of stock medium with or without agar (4 grams) to a 500 ml round bottom flask. Flushing the flask with 80:20 N_2:CO_2, heat the medium to boiling over a Bunsen burner. Heat the flask evenly and swirl the contents to keep the flask from breaking. Heat until the agar is dissolved. Cool it, and then add the reducing agent. The medium is reduced when the resazurin turns from red to colorless.

Step 2

Using the Hungate technique with 80:20 N_2:CO_2 flush the tubes and dispense the medium: 4.5 or 5 ml per Bellco tube or 30 ml per serum bottle. Close the vessel with a butyl stopper.

Step 3.

Cap the tubes or bottle and autoclave for 15 minutes.

Step 4.

Cool the tubes to 45-47° C in a water bath if agar is present.

Isolation and Serial Dilution of Methanogenic Bacteria in Roll Tubes

Materials:

Hungate gassing station
80:20 N_2:CO_2 gas mixture
80:20 H_2:CO_2 gas mixture
sterile pipettes
ice
stock culture of methanogenic bacteria

Using the Hungate Technique, make a serial dilution of a stock culture of methane bacteria. The manipulations will be made using 80:20 N_2:CO_2.

Make the roll tubes by placing the inoculated tubes in a horizontal position. While spinning the tubes in ice, the agar is solidified.

Gas the tubes with 80 percent H_2 and 20 percent CO_2. Incubate the tubes at 37° C until colonies appear. During growth, pressurize the tubes with 80:20 H_2:CO_2.

Picking Isolated Colonies from the Roll Tube Serial Dilutions

Step 1

Examine the roll tubes already made. Look for isolated colonies in the tubes at the greater dilutions. Using a sterile Pasteur pipette modified with a right angle bend near the tip, pick colonies from the roll tube while flushing it with 80:20 N_2:CO_2.

Step 2

Pick several colonies and inoculate the tubes of stock liquid medium with them. Flush the tubes with 80:20 H_2:CO_2, and incubate the cultures on their sides at 37° C. Pressurize the tubes with the hydrogen mixture every couple of days.

Isolation of Unknown Organisms

Repeat the media preparation and roll tube procedure but use the inoculum either from an enrichment or directly from the habitat.

For the isolation of a methanogen in Step 3 only examine tubes that indicate the presence of methane after gas chromatographic analysis.

Pick up and re-isolate unknown colonies repeating Step 2 three consecutive times.

After purity is assured, place the organism in liquid culture.

Stock Medium: per 200 ml

Medium B (Basal Medium)

170 ml distilled water
7.5 ml Mineral I
7.5 ml Mineral III
2 ml 20 percent YE - 20 percent TRY
3 ml 20 percent Na Formate
0.2 ml 0.1 percent resazurin
2 ml Wolfe's vitamins
2 ml trace minerals
0.8 g $NaHCO_3$
2 ml 25 percent Na Acetate

Medium M: per 200 ml
100 ml of 2X Basal
100 ml of distilled water

Medium MC: per 200 ml
100 ml of 2X Basal
10 ml of 20X TYC
90 ml of distilled water

Medium VM: per 200 ml
100 ml of 2X Basal
20 ml of 10X LIP
1 ml of 25 percent Na Acetate
50 ml of Mineral 4
30 ml of distilled water

Medium VMC: per 200 ml
as VM with the following.
10 ml of 20X TYC
20 ml of distilled water

Mineral 1 (g/l)

3.1 K_2HPO_4

Mineral 2 (g/l)

3.0 K_2HPO_4

Mineral 3 (g/l)

0.67 KCl
5.5 $MgCl_2 \cdot H_2O$

2X Basal (g/l)

5.0 $NaHCO_3$
2.5 NH_4Cl
100 ml of Mineral 1
50 ml of Mineral 2
20 ml of trace mineral
12.0 NaCl
2.4 $MgSO_4 \cdot 7H_2O$
0.8 $CaCl_2 \cdot 2H_2O$
6.9 $MgSO_4 \cdot 7H_2O$
0.5 NH_4Cl
0.28 $CaCl_2$
0.28 K_2HPO_4

Mineral 4 (g/l)

72 NaCl
0.67 KCl
1.4 $MgSO_4 \cdot 7H_2O$

20X TYC

2.5 percent Yeast Extract
5 percent Casamino Acids
0.05 percent Tryptophan

10X LIP (g/ml)

0.01 isoleucine
0.005 leucine
0.00005 pantothenic acid

Wolfe's Vitamins: (in 1 liter of distilled water stored in the cold and dark)

2 mg biotin	5 mg niacin	2 mg folic acid
5 mg Ca pantothenate	10 mg pyridoxine HCl	0.1 mg B_{12}
5 mg thiamine HCl	5 mg p-amino-benzoic acid	5 mg riboflavin
5 mg thiotic acid		

Trace Minerals: (in 1 liter of distilled water dissolve nitriloacetic acid with KOH to pH 6.5)

with KOH to pH 6.5	
1.5 g nitriloacetic acid	
0.5 g $MnSO_4 \cdot 4H_2O$	0.1 g $FeSO_4 \cdot 7H_2O$
0.1 g $CoCl_2 \cdot 6H_2O$	0.2 g $ZnSO_4 \cdot 7H_2O$
0.01 g g $CuSO_4 \cdot 5H_2O$	0.01 g $AlK(SO_4)_2 \cdot 12H_2O$
0.1 g H_3BO_3	0.01 g $NaMoO_4 \cdot 2H_2O$
0.05 g $NiCl_2 \cdot 6H_2O$	0.263 g $NaSeO_3 \cdot 5H_2O$

REDUCING AGENT

Boil 185 ml of distilled water in a N_2 atmosphere. Add 13.4 ml of 3N NaOH. Let cool. Add 0.5 g cysteine hydrochloride to water, mix, then add 2.5 g of $Na_2S.9H_2O$. Using the Hungate Technique, dispense 8 ml quantities of the reducing agent into tubes. Seal with the butyl stoppers and autoclave for 15 minutes.

CARBON ISOTOPE FRACTIONATION METHODS

Bomb Combustion Technique for Preparation, Analysis and *Delta* ^{13}C Results of Cell Material

Organisms from the field or from cultures can be centrifuged out of liquid media or field water. Treatment with excess 1.0 N HCl is useful to dissolve any carbonate granules present. The sample is then lyophilized to dryness. Dry cell mass is the desired material for *delta* ^{13}C mass spectrometric analysis.

Take samples with carefully washed forceps and bottles, and avoid contamination such as dust and lint. Weigh one to two mg of dry cell material into a carbon free silver (.999...) boat. Put the boat into a quartz tube with one end sealed and add approximately 40 mg of carbon free copper oxide. Pump the quartz tube (6 mm I.D.) down to 0-10 millitorr and seal it under vacuum.

Heat the tube to above 800° C and allow it to cool slowly. Halogens, nitrogen oxides and some other undesired compounds react with the silver; all cell mass is converted to CO_2.

Open the cooled quartz tube under vacuum through a methanol-dry ice trap to remove water. Cool the next trap to -196° C with liquid nitrogen and the CO_2 is caught (N_2 will go out). Continue trapping for 15 min. Measure the total CO_2 in a manometer. The measurement can be calculated in μM CO_2 per weight of sample. Solidify the CO_2 in a pyrex tube at -196° C with liquid nitrogen. Seal the solid CO_2 in the tube under vacuum.

Place the pyrex tube in the mass spectrometer port. Establish vacuum in the port, and open the tube. The CO_2 moves through the spectrometer to the detector. $^{13}CO_2$ weighs 45 A.M.U. The $^{12}CO_2$ weighs 44 A.M.U. The relative amounts of these are determined relative to a standard and corrected to PDB belemmite *delta* ^{13}C.

Isotopic Analyis of Liquids and Gases

Media, field samples

Collect water samples in the field (We did this at Embarcadero Road using test tubes subsequently closed to the atmosphere.) Place a known quantity of the sample (usually 0.5 ml) in a closed vessel, freeze and then evacuate the vessel. Melt the sample, and react it with a mixture of 1 ml 0.25 *M* $CuSO_4$ and 1 ml of 1 *M* H_2SO_4. The $CuSO_4$ effectively removes the dissolved sulfide species as insoluble CuS. The acid forces the dissolved carbonates out of solution and into the gas phase. Freeze the fluid again. Isolate and test the CO_2, now gaseous, both for quantity and isotopic composition.

Media

We followed a procedure similar to that above; differing only in that 2 ml of medium sample were used. Unfortunately, we allowed the media too much contact with air before sealing our samples. It is therefore likely that much CO_2 was lost, causing our measured CO_2 (aq) concentrations to be too low. It is also possible that the isotopic compositions are systematically in error. However, the parallel samples, especially 3a and 3b, 5a and 5b, and 9a and 9b, were exposed to the atmosphere for radically different times, yet were quite similar

isotopically, implying that the isotopic composition of the media did not have sufficient time to reach equilibrium. The result was that the samples were "frozen" at the original isotopic composition.

Delta ^{13}C Isotope Fractionation

To prevent closed system effects a system was devised to allow continuous bubbling of humidified 80:20 (v/v) mixture of H_2/CO_2 through the medium during incubation (see Fig III-10). Prior to inoculation the apparatus is autoclaved with media present, and then immediately removed. Gassing is then initiated using the filter sterilized gas mixture. To allow isotopic equilibrium to be established the medium is stirred and gassed overnight at incubation temperatures.

Sterilized reducing agent (see Methanogenic Bacteria Media and Techniques, above) is added aseptically by syringe immediately before inoculation. Two to four ml of an exponentially growing culture of *M. thermoautotrophicum* is inoculated into 100 ml of medium. The cultures are grown, at two temperatures, on modified M medium (Methanogenic Bacteria Media and Techniques) in which all nitrogen was supplied as $NH_4.2SO_4$ (3.08 g/l) to allow for later determination of the *delta* ^{13}C fractionation pattern.

For isotopic analysis gases are collected in sampling vessels from the media exit line. The incoming gas mixture is sampled directly from the cylinder. All gases are sampled immediately prior to inoculation and at harvest. For the measurement of total medium CO_2 a syringe is used to remove the liquid medium which is then rapidly injected into 5 ml sampling vials. Samples are stored frozen for analysis.

Cells are killed by aeration and harvested by centrifugation (10,000 xg for 10 minutes) at 4° C. The supernatant is decanted and the pellet sampled for total cell isotope composition. The remaining cells are extracted for total lipids using appropriate modifications of the method of Bligh and Dyer (1959), as described by DeNiro and Epstein (1978). Blank samples are processed concurrently and should show no significant noncellular materials (as determined by CO_2 after combustion). Cells, total lipids and lipid blanks are dried under vacuum, combusted in sealed quartz tubes and the resulting CO_2 isotopic composition measured using the methods of DesMarais (personal communication). The total medium CO_2 is determined by the addition of H_2SO_4 which releases all the carbonate present as CO_2. Carbon dioxide, cryogenically

trapped, is then processed as above. $CuSO_4$ was included in this treatment to remove sulfides present as reducing agents in the medium.

Methane production is measured after removal of CO_2 by cryogenic trapping. The methane is then combusted and the resulting CO_2 collected for isotope analysis.

Interspecies Hydrogen Transfer

We used special media for the growth of phototrophic bacteria:

BGM red, BGM^{o} red (Media are according to Rippka *et al.* (1977) but are pre-reduced according to the Hungate Technique described above.)

M/M
Medium modified from Medium M (see Hungate Technique) by using only 4 ml trace elements for making up the 2X Basal

BM/M
Medium containing 50 percent BGM^{o} and 50 percent M/M.

Combined Culture Media

VMC/M
Medium modified from VMC (see Hungate Technique) by replacing 20 ml LIP by water and by using a modified 2X Basal (50 ml mineral 1, 25 ml mineral 2, 4 ml trace elements instead of the normal amount). Further modifications of the VMC/M medium were: omission of mineral 4 and/or addition of $CaCO_3$ and/or the use of 1.25 percent cysteine instead of the reducing agent containing H_2S and cysteine.

Incubation Methods

Combined Culture

Perform all experiments in 55 ml rubber stoppered serum bottles, containing 20 ml medium and 35 ml N_2/CO_2 at atmospheric pressure. Incubate bottles in the light and at temperatures indicated in the text.

Separated Culture

Use an incubation system identical to that used by Giani and Krumbein (1982) except for the addition of a second cultivation tube (Fig. III-10). One of the culture vessels, contains BGM^{m}, the other one VMC/M. Reduce the VMC/M by adding 4 ml of 1.25 percent cysteine after gassing the system by N_2/CO_2 (95:5), closing it and pumping the gas-phase in a circuit to remove trace amounts of O_2 for about 1 hour.

BES Inhibition Experiment

Grow *Methanosarcina barkeri* in an MC medium with 0.4 ml of inoculum in 4.5 ml of medium. Gas one set of cultures with 30 psi H_2/CO_2 (80:20) daily, while the other set contains 0.1 *M* methanol (0.4 ml methanol/100 ml medium). Incubate the cultures at 37^{o} C in a water bath. Sample the headspace for methane and measure the optical density (ours was at 575 nm) at regular intervals. After 89 hours of slow growth, add varying concentrations of bromoethanesulfonate (BES) to the cultures and measure the headspace methane concentration at shorter time intervals. Make a stock BES solution (5 X 10^{-2} *M*) by dissolving 0.1065 g of BES (MW = 211) into 10 ml of boiled distilled water anaerobically. Make a series of BES solutions by serial dilution under anaerobic conditions, yielding concentrations of 5 x 10^{-3}, 5 x 10^{-4}, 5 x 10^{-5}, and 5 x 10^{-6} *M*. Sterilize the BES solutions by autoclaving. Add nine different concentrations of BES to the two sets of cultures by injecting either 0.1 or 0.5 ml of the various solutions into the two sets of cultures. Our final range of BES concentrations was 0.1 x 10^{-7}, 4.6 x 10^{-7}, 1 x 10^{-6}, 4.6 x 10^{-6}, 1 x 10^{-5}, 4.6 x 10^{-5}, and 1 x 10^{-4} *M*.

CHEMICAL METHODS AND MATERIALS FOR FIELD STUDIES WITH METHANOGENS

Sediment Methane Concentrations

Obtain sediment cores by hand, inserting a 60 cm PVC pipe (6.0 cm diameter) with side ports located at 3.0 cm intervals down its length. Seal the side ports with tape. Seal both the top and bottom of the pipe with rubber stoppers prior to removal. Sample the sediment immediately after core retrieval by removing the tape and inserting a cut-off 3 ml plastic syringe through the side port. Extrude the sediment aliquot into a pre-weighed serum vial (15 or 25 ml) containing 5.0 ml of 1.5 N NaOH. Quickly seal the vial with a black butyl rubber stopper, shake it to homogenize the contents. Prepare a blank by substituting distilled water for the sediment.

Analyze the samples for methane within 24-hours of the time the core was taken. Using a gas-tight syringe, inject 50-100 μl of headspace gas into a gas chromatograph (HP 5804A) equipped with a flame ionization detector and a 6 foot by 1/8 inch stainless steel column packed with Poropak Q. Prepare a series of standards by injecting known volumes of pure methane into capped serum vials containing a NaOH solution.

The detector response in our samples using this method was linear within the concentrations encountered. The standard deviation of 13 replicate injections was 1.3 percent. The presence of roots and leaves within the sediment can make it difficult to obtain a known volume of sediment. Thus, the headspace volume for each sample should be estimated from the mass of the sediment. We assumed a whole sediment density of 1.2 g/ml. We also estimated the methane concentration, reported as mmoles of methane per liter of interstitial water (*mM*), by assuming a constant sediment porosity of 0.8.

Total Dissolved Carbon Dioxide Concentrations

Extrude the core and section it into 2.0 cm slices. Place the sediment in plastic bottles and centrifuge them at 10,000 xg for 15 minutes. Immediately transfer duplicate 5.0 ml aliquots of the supernatant to small serum vials (total volume of 13.5 ml) and seal them with black butyl rubber stoppers. Freeze the samples until analysis.

Analyze the CO_2 using an HP5840A gas chromatograph with a 6 ft. by 1/8 inch Poropak Q column at 50° C and a Carle

microthermal conductivity detector (26° C, 25mA). Helium was the carrier gas with a flow rate of 50 cc/mm.

Prepare a primary CO_2 standard (1.0 *M*) by weighing 0.4233 g $NaHCO_3$ into a nitrogen-flushed serum vial, adding 5.0 ml of degassed distilled water, and sealing with a butyl rubber stopper. Make a secondary standard (50 m*M*) by diluting 0.5 ml of 1.0 *M* standard into 10.9 ml, also under nitrogen flushing conditions. Prepare a series of working standards by diluting 0.2 to 2.0 ml of the 50 m*M* standard to the following concentrations: 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 20.0 m*M* total CO_2.

Just prior to analysis, rapidly thaw the samples in a water bath and inject both standards and samples with 100 μl of 50 percent H_2SO_4 in order to convert all bicarbonate and carbonate ions to CO_2. Inject a sample of the headspace (100 μl) into the gas chromatograph. In our experiment a least squares fit of ths standards was linear with a correlation coefficient of 0.997. The standard deviation of duplicate samples was 3-9 percent.

Interstitial Water Sulfate Concentrations

Separate interstitial water from the sediment by centrifugation (4000 xg for 5-10 minutes) and filter the supernatant (0.2 μm Millipore filter). Determine the sulfate concentration turbidometrically as described in *Standard Methods for the Examination of Water and Wastewater* (1971). (Briefly: dilute the interstitial water (2.0-5.0 ml interstitial water per 100 ml distilled water), acidify it, and precipitate the sulfate by adding $BaCl_2$ crystals.)

We measured the absorbance of the $BaSO_4$ precipitate at 420 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. The standard deviation for this method is about 10 percent.

Interstitial Water Acetate Concentrations

Determine the concentration of acetate in the interstitial water by a specific enzymatic assay described by Cappenberg (1974). In the presence of ATP and acetate kinase, acetic acid is quantitatively phosphorylated to acetylphosphate. At pH 7, the acetylphosphate will react with hydroxylamine to form acethydroxyamic acid. This compound forms a complex with $FeCl_3$ that absorbs at 540 nm.

Most Probable Number of Methanogenic Bacteria

We conducted a most-probable-number (MPN) study on sediment samples from the Palo Alto Baylands salt marsh to determine indirectly the number of methanogens as a function of sediment depth (*Standard Methods for the Examination of Water and Waste Water*, thirteenth edition, 1971).

Sediment samples from various depths are inoculated into two media; VMC medium containing 14 m*M* sodium acetate with a H_2/CO_2 (80:20) gas atmosphere (VMC-H_2/CO_2), and VMC medium containing 1.0 m*M* trimethylamine (TMA) with a N_2/CO_2 (80:20) gas atmosphere (VMC-TMA). Inoculate the samples anaerobically according to the technique described by Hungate (see above for a description of VMC medium and Hungate technique).

Serially dilute the samples to 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} x initial. The initial 10^{-1} dilution is made sediment into 4.5 ml of VMC media. The VMC-H_2/CO_2 dilutions were done in triplicate, with the VMC-TMA dilutions in duplicate.

Incubate the samples at 30° C for 7 days. Sample a headspace volume of 200 μl for methane determination as previously described.

Methanogen Enrichments

Extrude the sediment core and section it into 2.0 cm segments. Use a cut-off 3 ml syringe to subsample by inserting it vertically into the sediments and removing approximately 2.5 ml of sediment. Place two aliquots (for a total of 5.0 ml) from each sediment depth into a serum vial (total volume of 158 ml) while continually flushing it with N_2/CO_2 (80:20). Make a slurry by adding 50 ml of degassed distilled water to the serum vial. Enrich each set of sediment samples on one of the following substrates:

30 psi H_2/CO_2 (80:20)

10 μl 14 percent trimethylamine (TMA) for a final concentration of 0.25 percent (first experiment, 7/8/82), or 40 ml 25 percent TMA for a final concentration of 0.1 percent (second experiment, 7/15/82).

10 μl methanol for a final concentration of 0.1 percent.

40 μl 25 percent sodium acetate for a final concentration of 0.1 percent.

Seal the serum vials with black butyl rubber stoppers. Determine the methane concentration in the headspace approximately every 8 hours by gas chromatographic analysis as previously described (Methanogenic Bacteria Media and Techniques). Incubate the enrichments at 37° C for the duration of the experiment.

Sediment Methane Production Rates

Total Methane Production Rate: Jar Experiment

Take a core by inserting a 6.0 cm diameter PVC pipe into the salt marsh sediment. In the laboratory, extrude the upper 2.0 cm of sediment and then discard it. Use cut-off 3 ml plastic syringes to anaerobically transfer 2.5 ml sediment samples to 25.5 ml serum vials containing 1.0 ml of degassed marsh water. By extruding and discarding the previously sampled sediment, we obtained aliquots from 6 depths. Seal the vials with black butyl rubber stoppers and vortex them to form a homogeneous slurry Autoclave one set of replicates to provide a control.

Allow the sediment slurry to incubate at room temperature for 6.5 days. Since methane production causes a net decrease in pressure (4 *M* H_2 and 1 *M* CO_2 per 1 *M* CH_4 formed), pressurize the samples to 5 psi with a gas mixture of N_2/CO_2 (80:20) just prior to sampling. Also pressurize the methane standard to correct for the effect of pressure on the methane analysis. Using a "pressure-lok" syringe, analyze 0.1 ml of headspace gas for methane as previously described. Determine the methane production rate from the difference between the methane accumulated in the headspace of the sample and the autoclaved control (nmoles methane per gram of sediment) divided by the incubation time.

Methane Production Rates from $^{14}CO_2$

We designed a radiocarbon experiment to investigate the rate of methane production from CO_2. Prepare sediment slurries from the same core and the same depths as the previously described jar experiment. Treat the samples identically to those of the jar experiment, except that 50 μl of

^{14}C-bicarbonate solution (380,000 cpm) should be injected into each serum vial. Prepare controls by acidifying one set of replicate samples (pH 1) and immediately freezing.

Incubate the samples at room temperature for 6 days. Stop the incubation by adding 1.0 ml of 1 N NaOH which raises the pH to 11, thus inhibiting methanogenesis and trapping the CO_2 as carbonate ions. Collect the ^{14}C(methane) and $^{14}C(CO_2)$ separately using the stripping lines diagrammed in Figures III-9 and III-10. Collect the methane first by stripping it out of the alkaline sediment slurry, through a CO_2 trap, combusting it over copper oxide at 800° C, and trapping the resulting CO_2 in a phenethylamine based scintillation cocktail. The nitrogen carrier gas flow was 15-20 ml/min so that the methane resides in the furnace for 10 seconds. This is adequate time to completely combust greater than 99 percent of the methane (David DesMarais, personal communication). Strip the samples for 30 minutes so that the headspace flushes a minimum of 30 times. After removal of the methane, acidify the sample (pH 1) and strip the CO_2 to form the slurry. Collect the slurry in the phenethylamine scintillation cocktail. Trap the hydrogen sulfide using $CuSO_4$ adsorbed onto the surface of Chromsorb (an inert support). The flow rate should be about 50 ml/min.

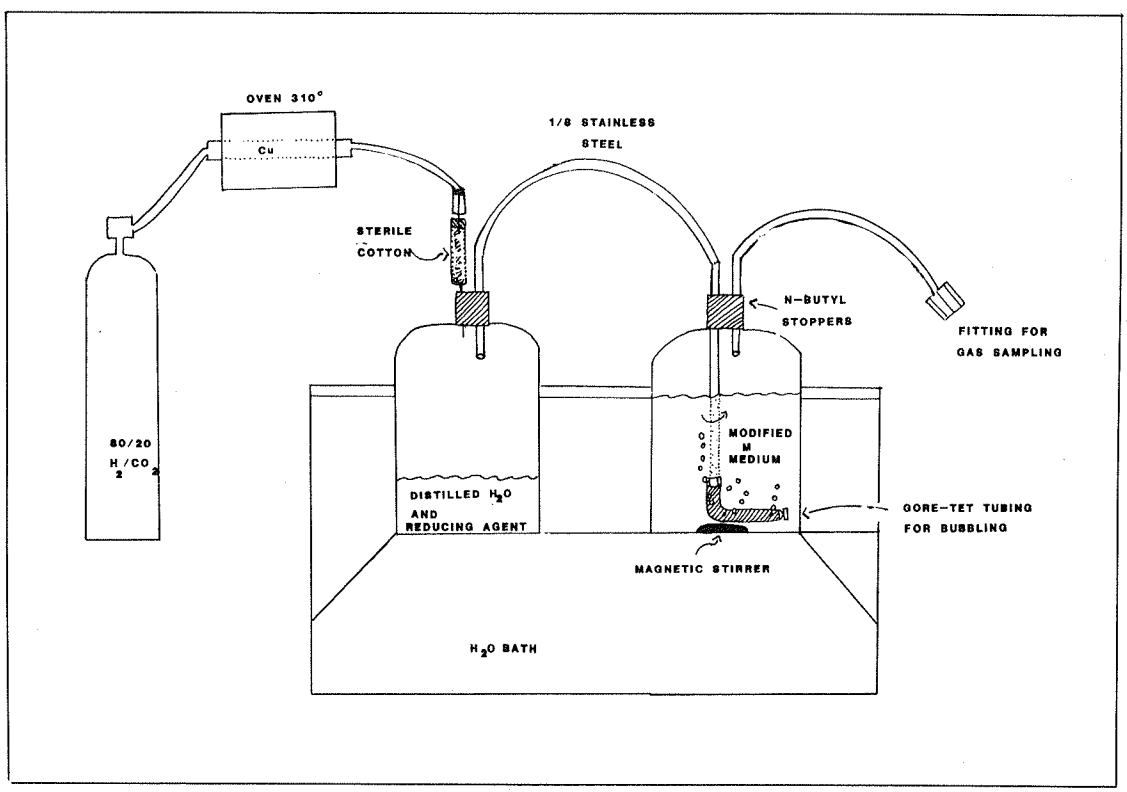

Fig. III-10 **Continuous flow system for culturing *Methanobacterium thermoautotrophicum***

Quantify the activity of methane and CO_2 from each sample on a Tri-Carb liquid scintillation counter. We assumed all our samples were equally quenched and no correction was made for counting efficiency. Thus all results are reported as counts per min. In our experiment the label recovery ranged from 75-85 percent while stripping the methane gas. The alkaline slurry would foam and creep out of the serum vial, possibly accounting for the lost radiocarbon. The methane production rate from bicarbonate can be calculated from the following equation:

$$\text{Rate} = \frac{(\text{total } CO_2) \times a}{A \times t}$$

The concentration of CO_2 is *mM*, A is the activity of the added ^{14}C-bicarbonate, *a* is the activity of recovered methane, and t is the incubation time.

Methane Production from Acetate and Methionine

Using techniques identical to the ^{14}C-bicarbonate experiment we investigated methane production from acetate and methionine. To do this, we injected sediment slurries from five depths with 50 μl of 2-^{14}C-acetate or methyl-labeled ^{14}C-methionine. After homogenization, the concentration of ^{14}C-acetate was 7 μM and ^{14}C-methionine was 0.15 μM.

After a 12 hour incubation, methane and CO_2 were stripped from the serum vials as previously described. No attempt was made to quantify the portion of substrate that may have been assimilated.

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APPENDIX IV
METHODS FOR STABLE CARBON ISOTOPIC ANALYSIS

(David DesMarais)

The experiments produced three types of samples for carbon isotopic analysis. These were: lyophilized cell material, aqueous inorganic carbon from culture media, and methane gas. The first three sections below describe how each of these samples was converted to purified carbon dioxide. The last section describes briefly the mass spectrometric isotopic analysis of the carbon dioxide.

Cellular Material

Combust lyophilized cell material to carbon dioxide by the bomb combustion method.

Materials

20 cm long by 9 mm O.D. quartz tube, flame sealed at one end; 0.5 g. of rodform cupric oxide; a sample boat fabricated from a piece of silver foil which is 0.25 mm thick and 7.5 cm long by 1.3 cm wide.

Preclean these materials by heating in an 850° C oven in an oxygen or air atmosphere. Add approximately 5 mg of moist cell material to the silver boat, and with the silver boat inside a covered Petri dish, dry them in a vacuum desiccator. Then place the boat and sample inside the quartz tube along with the 0.5 gram of cupric oxide. Evacuate the quartz tube and its contents to 10^{-5} torr or less on a vacuum line and seal it using a torch. Heat the tube, now termed a "bomb", in a 850° C oven for two hours. Allow it to cool slowly overnight to room temperature. The next day attach the bomb to the vacuum system (Fig. IV-1) at point B in order to purify and measure the carbon dioxide produced during the combustion. Crack the bomb open into the vacuum system (DesMarais and Hayes, 1976) and the carbon dioxide-water-nitrogen mixture will pass through valve 4. Traps C and E (chilled using dry ice-acetone) remove the water. The remaining gasses pass through valve 6 and the carbon dioxide is trapped in cold trap F (chilled using liquid nitrogen). Pump the nitrogen away to vacuum via valves 7 and 1. Transfer the carbon dioxide cryogenically to mercury manometer H for measurement and, subsequently, to 6 mm pyrex tubes located at 5. Seal the gas in the tubes using a torch.

Dissolved Inorganic Carbon

Sample the culture medium using a 3 ml disposable syringe immediately upon harvesting to avoid carbon losses due to outgassing. Then inject the 3 ml aliquot into a sealed Wheaton bottle which can be frozen if the sample is to be stored prior to analysis. Fit the vacuum line (Fig. IV-1) at point B with a "multiple" cold trap consisting of four loops of 1/8 inch O.D. stainless steel tubing. Each loop is oval and is 20 cm long by 3 to 4 cm wide. Attach one end of the trap to point B and the other end to a valve connected to a syringe needle (22 gauge). Thaw the Wheaton bottle and its sample. Inject 3 ml of a solution of 1 *M* H_2SO_4 and 1 *M* $CuSO_4$ in distilled water into it. Shake the bottle to allow the acid to liberate the carbon dioxide from the solution. Then attach the bottle to the vacuum line by penetrating its rubber stopper partway with the 22 gauge syringe needle. Freeze the bottle's contents using a dry ice-methanol bath. After the needle and vacuum line are pumped out, cool the multiple trap attached at point B using liquid nitrogen. Pump the gaseous contents of the Wheaton bottle through the multiple trap by pushing the needle completely through the stopper. The carbon dioxide and water are trapped in the multiple trap whereas the air is pumped away. After the air is evacuated, close the valve adjacent to the syringe needle, and process the frozen gases within the multiple trap in the same fashion as was described above for the gaseous products of the bomb combustion.

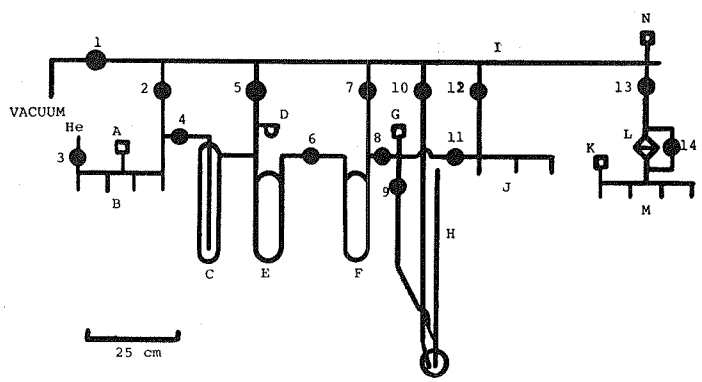

Fig.IV-1 Schematic Vacuum Line

Schematic diagram of vacuum line used in this project. The vacuum system consists of a liquid nitrogen-cooled trap in series with an Edwards Model EMI diffusion pump and a Welch Model 1400 mechanical vacuum pump. Numbers in the Figure refer to the valves in the system. The letters denote other components as follows: A, G, K, N-thermocouple pressure gauges; B-manifold for introducing samples (helium gas is swept in through valve 3 to keep this area clean when it is exposed to the atmosphere); C - glass cold trap; D - small mercury V-tube manometer; E, F - cold traps consisting of four loops of 1/4 inch O.D. stainless steel tubing; H - mercury closed-end manometer; I - vacuum manifold; J - manifold for 6 mm tubing to collect purified carbon dioxide for isotope analysis; L - coarse frit assembly; M - manifold used for evacuating and sealing bombs prior to combustion (frit L prevents ample particulates in the bombs from entering manifold I). Note 25 cm scale at lower left.

Methane

Combust the methane and purify the carbon dioxide using a gas chromatography-combustion (GCC) system similar to the one described by Matthews and Hayes (1978). Sample and store the methane in a 125 ml Wheaton bottle. Then inject the methane into the GCC via a Valco 8-part valve. Purify the methane using a Porasil B column (1/8 in. x 180 cm stainless steel tubing packed with 80-100 mesh Porasil B and operated at 25° C with a 15 cc/min helium carrier gas flow). Combust the purified methane as it flows through a 780° C oven packed with 60-100 mesh cupric oxide. After the carbon dioxide is trapped, purify it by cryogenic distillation (-135° C) from a variable temperature trap (Des Marais, 1978).

Isotope Mass Spectrometry

Analyze the carbon dioxide samples using a Nuclide 6-60 RMS mass spectrometer fitted with a special inlet system which enables small (as little as 0.05 μM) samples to be analyzed to a precision typically exceeding 1 $^{o}/_{oo}$ (0.1 $^{o}/_{oo}$ is attained with 0.5 μM samples). Inlet systems of this type are discussed by Hayes *et. al.* (1977).

APPENDIX V
CARBON ISOTOPE DATA INTERPRETATION

The stable carbon isotope studies conducted in the 1982 NASA FBME Summer Research Program provided ample opportunity to judge the power as well as the limitations of the techniques and approaches used. We include these comments at the end of this report because they can be equally well applied to all three chapters.

First, it is clear that stable isotope fractionation can be used as an indication of biological processes: carbon fixation and metabolism. The method provides a potentially powerful tool for the understanding of microbial chemistry and microbial interactions in culture and in nature. Even without a complete understanding of the entire system, (environment, organisms, etc.) these techniques can be used to formulate key statements regarding the role of various types of biota in the cycling of organic carbon.

On a more cautious note, we have learned that careful attention must be paid to the uncertainties involved in the systems under study. If the techniques are to be valuable as ecological tools, then the factors that could disturb the system should be measured and known in as much detail as possible. Some of the factors that can exert significant influence on isotope fractionation are:

1. What organism(s) is/are present (and its/their carbon metabolic interactions)
2. Carbon metabolic pathways
3. Physiological state of the organisms
4. Rate of carbon substrate consumption, growth, and cell densities
5. Cellular turnover of incorporated carbon
6. Isotopic values of carbon compounds in the environment
7. Sinks of carbon (intra and extracellular)
8. Culture methods, including supply of carbon containing metabolites
9. Concentration and fluxes of extracellular sources of carbon

Clearly with pure and eventually mixed culture studies of the kind reported, we can begin to unravel the complexity of stable isotope fractionations, leading to reproducible observations and interpretable measurements. Until this is achieved, it will be difficult to use the carbon fractionation approach to unambiguously identify microbial communities, their environments, and metabolic processes. The observation that seems certain is that the extent of fractionation is determined by biological processes strongly responsive to key environmental variables. Thus, a major research goal is to develop a bank of reliable data in pure culture, in mixed cultures, and also in natural samples taken from the field. We feel that the PBME 1982 Summer Program was a significant step in the achievement of these goals. One of the most notable advances has been the establishment of a variety of collaborations between stable isotope geochemists and microbiologists; these have led to many studies in which organisms under a variety of conditions and defined stages can be examined and the data accumulated.

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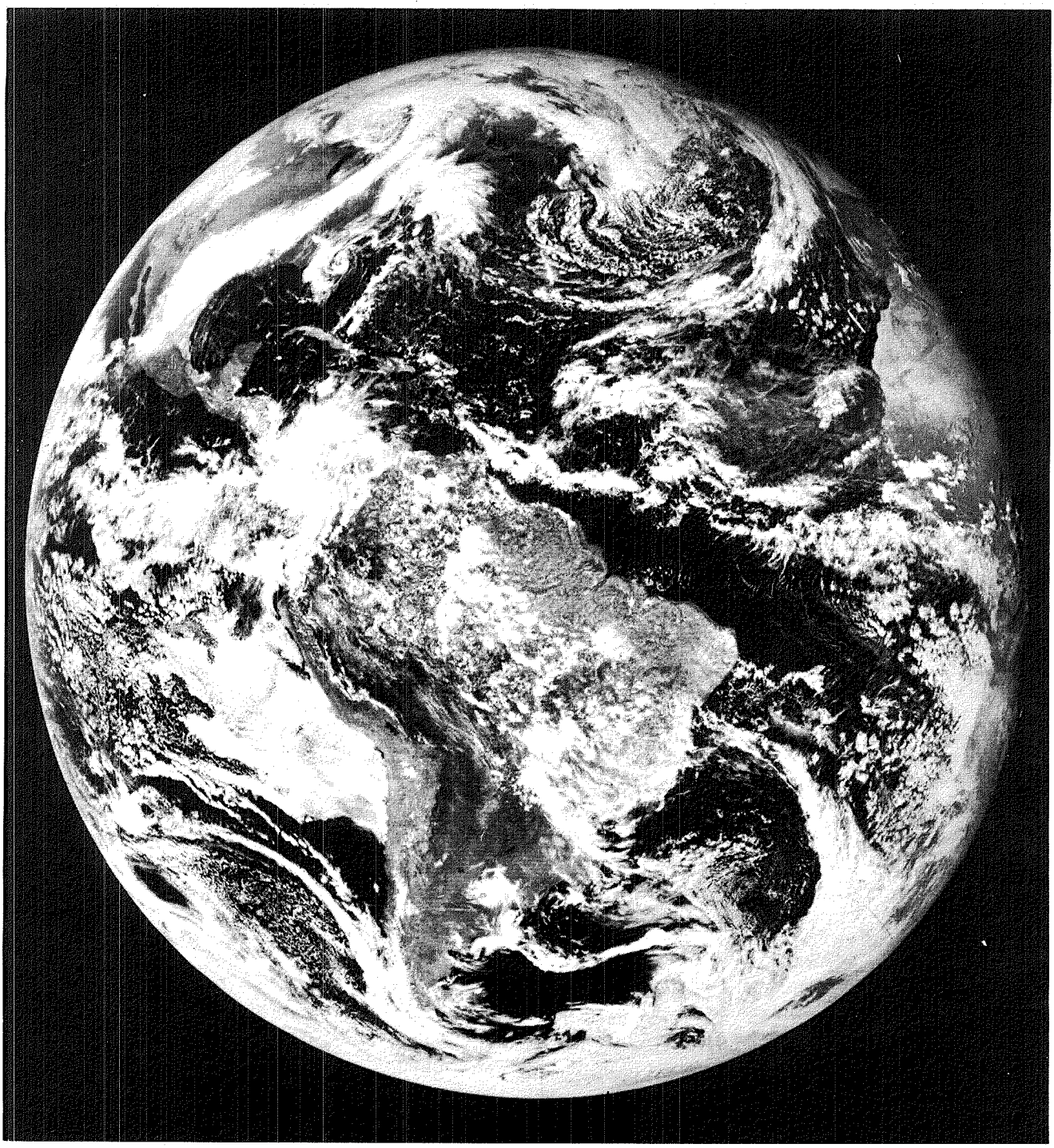
INDEX



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16. Abstract

This is a report of the proceedings of the 1982 Planetary Biology and Microbial Ecology research group that converged and studied in Santa Clara. The research group consisted of scientists and students. The body of this report details experiments made with cyanobacteria, phototrophic bacteria, and methanogenic bacteria. Significant carbon isotope fractionation data is included. Taken from well documented extant microbial communities this data provides a basis of comparison for isotope fractionation values measured in Archean and Proterozoic (preCambrian) rocks. Media, methods, and techniques used to aquire data are also described.

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